The use of piscine innate immune responses as indicators for environmental pollution in marine ecosystems

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Contents

Abbreviation list

ACHE	:acetylcholin esterase
AhR	:aryl hydrocarbon receptor
aqua dest.	:Aqua destilatum (distilled water)
CF	:condition factor
CYP450	:cytochrome P450
DDD	: 1,1-dichloro-2,2-bis(p-chlorophenyl) ethane
DDE	: 1,1-dichloro-2,2-bis(chlorophenyl) ethylene
DDT	: 1,1,1-trichloro-2,2-bis(p-chlorophenyl) ethane
DHR	:di-hydrorhodamin
DMSO	:dimethylsulfoxide
DNA	:desoxyribonucleic acid
EROD	:Ethoxyresorufin-O-deethylase
et al.	:et alii (and others)
FACS	:Fluorescence accelerated cell scanner
FITC	:flourescin-iso-thio-cyanate
FSC	:forward scattered light (reflecting cell size)
G6PDH	:glucose-6-phosphate dehydrogenase
GST	:glutathione S-transferase
H_2O_2	:hydrogen peroxide
HCB	:hexachlorobenzene
HCH	:hexachlorocyclohexan
HKL	:head kidney leukocytes
ICES	:International Council for the Exploration of the Sea
IU	:international unit
MAA	:macrophage aggregate area
MAM	:macrophage aggregate activity
n	:number of samples
NBT	:nitro blue tetrazolium salt
NR	:neutral red
O_2^-	:superoxide radical
OD	:optical density
p	:probability of error

PAH	:polycyclic aromatic hydrocarbon
PBL	:peripheral blood leukocytes
PBS	:phosphate buffered saline
PCB	:polychlorinated biphenyls
PMA	:phorbol-12-myristate-13-acetate
ppm	:parts per million
R	:coefficient of correlation
ROS	:reactive oxygen species
RPMI 1640	:Roswell Park Memorial Institute cell culture medium No 1640
SI	:stimulation index
SSC	:side scattered light (reflecting cell complexity)
v/v	:volume per volume

Abstract

Abstract

Environmental pollution especially in aquatic ecosystems is a major problem of the 20th century. Industrial effluents mainly introduce a wide variety of xenobiotics into marine or limnetic ecosystems which can enter aquatic organisms by different routes, interact with metabolic pathways or act direct as toxicant. These substances finally induce stress, which results in limited distribution, low abundance and /or reduced reproductive potential of affected organisms and therefore can induce changes in the entire ecosystem. An attempt to assess the changes induced by environmental contaminants is to monitor so called biomarkers or bioindicators: molecular, cellular or physiological parameters of organisms which are modulated in response to xenobiotic challenge. These parameters in principle should be measured by simple and inexpensive techniques, they should be sensitive to sublethal concentration of xenobiotica in the environment, and when measured in biota they should indicate the effect of pollution on the organism. In the research presented here, we incorporated innate immune responses of flatfish into biological effect monitoring studies to assess the immunomodulatory influence of xenobiotics. An effect of single compounds or of mixtures of xenobiotica on innate immune response of fishes was previously shown in laboratory studies by others.

As a prerequisite, we showed that the head kidney of flounder (*Platichthys flesus* L.) is the optimal target organ for the conduction of functional assays such as the generation of radical oxygen species (ROS) or endocytosis by granulocytes and macrophages/ monocytes. Head kidney derived leukocytes gave highest measurements when compared to cells from blood or spleen, even without further enrichment protocols. Thus, these cell suspensions can be used “under field situations” (chapter 2).

When considering these innate immune responses as biomarkers or indicators for environmental degradation, pollution mediated effects have to be distinguished from natural fluctuations. The natural impact of hydrographical factors for instance can modulate immune responses in fish. As shown in this thesis (chapter 3) a decrease in ambient salinity from 32 to 16 ppm did not result in a redistribution of leucocyte subsets in the head kidney of the euryhaline flounder. Phagocyte functions of head kidney derived leucocytes, such a respiratory burst and pinocytosis activity as well as plasma lysozyme levels also were not altered upon the change in salinity. The findings here indicate that these parameters are not sensitive to salinity changes in brackish or estuarine environments.

The infection of flounder with different parasites did not result in alteration of innate immune response (chapter 5). Due to a high variability in infection status in addition to a high variability in immune functions, no dependencies were obvious. Our findings indicate that ectoparasitic copepods as the most abundant parasites had no major influence on the immune responses measured here, which means, that these parameters are not sensitive to sub-clinical parasite infection.

In an integrated biomonitoring study (chapter 4) conducted in the German Bight, southern North Sea, ROS production and lysozyme activity in flounder were significantly affected by single xenobiotica and significant correlations of the immunological parameters applied here were found with ICES recommended biomarkers of xenobiotica exposure as EROD or DNA unwinding. Due to the moderate pollution gradient found in the German Bight no spatial trend between the sampling sites could be drawn by means of the immunological parameters.

In an integrated study on dab (*Limanda limanda* L.), where a clear pollution gradient between the sampling sites had been described, the ability of granulocytes and macrophages/monocytes to generate ROS was lower in polluted sites. Lysozyme activity in blood was significant lower in polluted sites and also in individuals infected with the lymphocystis virus or with nematodes (chapter 6).

The present study underlines that the chosen immune assays could be used in biological effect monitoring studies under field situations and can show a general modulatory effect of xenobiotica on fish immune responses under natural conditions.

Chapter 1

General Introduction

General introduction

About 70 % of the earth's surface and more than 90 % of the earth's biosphere is water. The aquatic environment is perceived to be at risk of several thousands of toxic chemicals, of both anthropogenic and natural origin. If they are not brought into the environment deliberately, hydrologic and atmospheric processes will disseminate these chemicals, referred to as xenobiotics, eventually depositing them into the aquatic ecosystem. As a result of the industrial revolution, more than 100 000 xenobiotic compounds are in commercial use in the 20th century (Depledge and Fossi 1994), and the ultimate sink for many of them is the aquatic environment. Many chemicals are no longer produced but nevertheless persist in the environment, and several hundreds of new chemicals are still introduced each year (Moriarty 1993). The xenobiotics (table 1) can enter the organisms by different routes, interact with metabolic pathways or act direct as toxicant and finely induce stress.

Table 1: Ecotoxicants, which have been studied because of their potential to harm fish's innate immune system (modified after Bols *et al.* 2001). Groups based on two properties: biological activity and chemical structure.

Biological activity
INSECTICIDES (TOXIC TO INSECTS) (subdivided on basis of chemical structure):
• Organochlorines:
mirex, kepone, toxaphene, endosulfan, lindan, hexachloro-cyclohexane
• Organophosphates:
trichlorfon, dichlorvos 1-napthyl-N-methylcarbamate (carbaryl or methacid-50), malathion, methyl parathion
• Pyrethroids:
esfenvalerate
HERBICIDES (toxic to plants):
atrazine
FUNGICIDES (toxic to fungi):
chlorothalonil
ENVIRONMENTAL ESTROGENS (bind to estrogen receptor):
nonylphenol, nonoxynol

Table 1 (continued):

Chemical structure
POLYCYCLIC AROMATIC HYDROCARBONS (PAHs): benzo[a]pyrene (BaP), 3-methylcholanthrene (3MC), 7,12-dimethylbenz[a]anthracene (DMBA) Dioxins and furans 210 congeners; two are 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDF) and 2,3,7,8-tetrachlorodibenzofuran (TCDD)
POLYCHLORINATED BIPHENYLS (PCB): arochlor is a commercial PCB mixture, 209 possible congeners
PHENOLS: phenol, pyrocatechol, hydroquinone, 2,4,6-trichlorophenol, pentachlorophenol (PCP)
HEAVY METALS: cadmium, chromium, copper, lead, mercury, manganese, nickel and zinc Organometallics: tributyltin, dibutyltin (TBT, DBT)

A population under stress due to some anthropogenic changes in its environment is frequently characterised by a limited distribution, low abundance and /or reduced reproductive potential. Thus, the ultimate expression of stress at the population level is a decrease in the absolute number of adults (Diamant and Westernhagen 1999) and on ecosystem level, a decline in biodiversity.

To asses the impact of pollution on the environment, the xenobiotica load is correlated with contamination found in biota or organisms. Different methods have been introduced and applied in the field to monitor the xenobiotica load. Chemical monitoring focuses on a selected set of well-known contaminants, which are measured in abiotic environmental compartments like sediment or water, while bioaccumulation monitoring focuses on contaminant levels in biota. The effect of xenobiotica introduced into the environment on biota is difficult to assess. Both methods cannot answer the question of consequences for the biota which arise with the contamination load. Consequences at the ecosystem level normally display a long response time, and when effects eventually occur, it is usually to late for effective countermeasures to be taken. In addition interactions between different xenobiotics are also difficult to assess, but there are attempts to measure an overall effect of pollution, for instance by infection experiments with opportunistic pathogens.

In biological effect monitoring early adverse alterations that are partly or fully reversible (biomarkers) are determined in biota. A biomarker is defined as a sublethal biological measurement of response to, or effects of, pollutants in living organisms (Peakall 1994). These include a number of molecular, cellular and physiological parameters that, in principle, can be measured with simple and inexpensive techniques. Biomarkers have been identified as a powerful and cost-effective approach to obtain information on the state of the environment and the effects of pollution on living biological resources (McCarthy and Shugart 1990, Peakall 1994, Wester *et al.* 1994, Depledge *et al.* 1995). By the application of biomarker measurements, the use of expensive and complex analytical chemical equipment and expertise can be reduced, and analyses are relatively quick to perform. The response of these biomarker systems represents a sublethal response to toxic compounds, thus providing not only early warning indicators of degradation in environmental quality, but also specific measures of the biological availability of toxic, carcinogenic and mutagenic compounds in the environment (Goksoyr *et al.* 1996). The sequential order of responses to pollutant stress is visualised in Figure 1. Responses at higher levels (such as tissues, organisms) are obviously late effects following long term exposure, whereas early effects of pollution can be observed up to cellular level. For example biotransformation enzymes and products, oxidative stress parameters, stress proteins and metallothioneins, as well as haematological, immunological, reproductive, endocrine, genotoxic, physiological and morphological parameters provide biomarkers for early stages in stress responses (Van der Oost 1998).

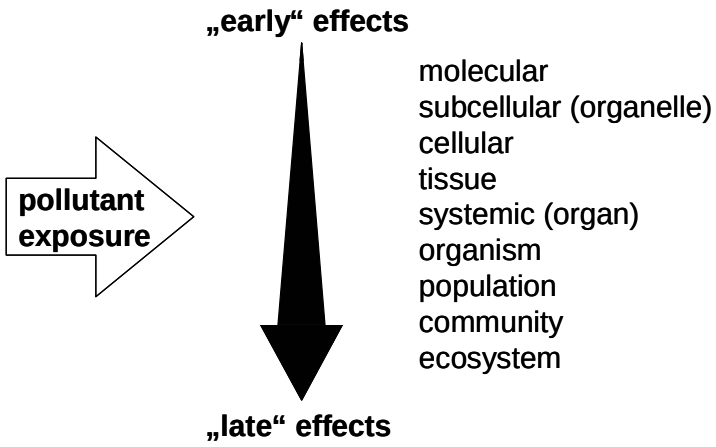


Figure 1: The sequential order of responses to pollutant stress within a biological system. Modified from Bayne *et al.* (1985)

It appears clear from a number of studies that the immune system is exquisitely sensitive for assessing toxic effects of chemicals of environmental concern (Luster *et al.* 1988, Luster and Rosenthal 1993). Because of the responsivity of the immune system to chemicals at low levels and its importance for maintaining host resistance against disease, much emphasis has placed on the development of immune assays that can predict exposure to and/or effects from environmental chemicals (Zelikoff *et al.* 2000). These assays, originally developed in rodents, have been adapted for use in a variety of animal species (Weeks *et al.* 1992) including fish. The immune system in general can be divided into the innate immune system, which responses immediately and independent from previous exposure from a particular antigen, and the adaptive immune system, which responses upon antigen exposure. Among the entire immune system of a multicellular animal that can be monitored in ecotoxicology, the innate immune system has some unique attractive features. More than other biological processes, the innate immune system is directly concerned with the interaction of a species with other organism- an essential feature in ecology. Detrimental changes to innate immunity are of ecotoxicological concern because they have the potential to influence populations by affecting susceptibility of individuals to disease. Additionally, many components of the innate immunity appear to be evolutionary conserved (Hoffmann *et al.* 1999, Ulevitch 2000). This could mean that the sensitivity of innate immune mechanisms to a particular contaminant is similar among species, which would make predicting the environmental impact of toxicant easier.

Fish are an especially important animal group from the perspective of innate immunity and ecotoxicology. Impairment of innate immunity may be more significant in fish than in mammals, as mounting an adaptive or acquired immune response takes longer in fish (Alexander and Ingram 1992). Understanding changes in fish innate immunity are important for evaluating changes in the general health of the aquatic environment. Fish are the oldest and most diverse of the vertebrate groups and consist of more than 25,000 different species occupying most aquatic niches. Thus, monitoring fish health gives insight into the condition of the aquatic environment (Zelikoff *et al.* 2000). An effect on fish innate immunity can serve as a warning of potential impact on human and ecosystem health because ecotoxicants (Table 1) are often released first into aquatic environments, an then, by a variety of routes, reach humans and other terrestrial animals (Zelikoff *et al.* 1998, Adams and Greesly 1999).

The immune system of fishes is quite varied and appears to be associated with fish phylogeny (Borysenko 1976). Although even the most advanced teleost species do not possess bone marrow or lymph nodes, fish contain functionally equivalent haematopoetic tissue primarily in areas of the kidney, spleen, and thymus. In addition, fish also have circulating white blood cells that are functionally and morphologically similar to mammalian lymphocytes, granulocytes, and monocytes (Ellis 1977, Zelikoff *et al.* 1991, Enane *et al.* 1993). Fish and mammalian species share a number of structural and functional characteristics important in the humoral, cell-mediated, and non-specific aspects of immune response (for review: Zelikoff 1994). Non-specific immune reactions in fish are general responses to injury and/or invasion by foreign organisms. As in their mammalian counterparts, phagocytosis and inflammation are two non-specific responses that appear to be universal in fish (Corbel 1975). Macrophages, along with neutrophils and non-specific cytotoxic cells (NCC), are the principal cell types associated with carrying out non-specific immune reactions in fish. White blood cell responses are assisted and/or heightened by a variety of non-specific factors found in fish serum, including complement, lysozyme, interferon, transferrin, C-reactive proteins and various natural haemolysins and haemagglutinins (Corbel 1975).

One important fish group of indicator species for biomonitoring studies is represented by the flatfish family Pleuronectidae, living in close contact with and feeding on sediments. The two flatfish species applied in this study, the European flounder (*Platichthys flesus* L.) and dab (*Limanda limanda* L.), are widespread and prevalent in high abundance in the area and thus fulfil the criteria for an indicator species in biomonitoring studies. The flounder is common in coastal zones and estuaries up to freshwater areas in rivers in the North Sea and Baltic Sea. It typically prefers finegrained to sandy sediments, where it feeds mostly on benthic invertebrates. Most of the year, the flounder is a rather stationary species. The dab is also used as a monitoring organism in the North Sea and Baltic Sea, due its abundance, wide distribution, and susceptibility to environmental stressors (Lang and Dethlefsen 1996). Like the flounder, it also prefers finegrained to sandy sediments and feeds mostly on benthic invertebrates. Both species do migrate into deeper waters during the winter months for spawning and return to the same shallow waters during the summer period, which they left in winter. Therefore they have been applied in a variety of national and international monitoring programmes. For ecotoxicological studies the available data base on flounder and dab includes both baseline biological studies as well as studies on fish diseases and pollution

bioaccumulation and their physiological and pathological effects (Gokskoyr *et al.* 1996, Dethlefsen *et al.* 2000).

The aim of the present investigation was to integrate innate immune responses of flatfishes as biomarkers into existing biological effect monitoring studies. Studies which correlate measurements of innate immune responses with data of somehow more established biomarkers of exposure such as EROD, Cytochrome P450 activity, DNA breakages or with bioaccumulation data from the same individual are scarce and in combination with long time monitoring studies they are novel. But this kind of studies offer additional information on the influence of xenobiotica at an individual level, about spatial or temporal patterns of response and might open the way to a multidimensional analysis (van der Oost *et al.* 1997).

Here we chose those innate immune assays, which were held as the most promising for biomarkers due to results derived from exposure studies conducted in the laboratory, mesocosms or under field situation (for review: Bols *et al.* 2001). This were the ability of phagocytes to conduct endocytosis and to generate radical oxygen species (ROS), as part of the first line of defence in fish against pathogens, as well the activity of lysozyme in blood and the packed red blood cell volume. These parameters showed sensitivity against pollution and were influenced by xenobiotics (Bols *et al.* 2001). These innate immune parameters fulfil the criteria for biomarkers such as applicability in field situations, cost effectiveness, the use of no special/expensive equipment, a high trough put and the analysis can be performed relatively quickly. In an attempt to incorporate measurements of these innate immune responses into an integrated biological effect monitoring concept in flatfish, leucocytes initially were isolated from peripheral blood, head kidney and spleen and analysed for the presence of different leucocyte subsets, which were responsible for the phagocyte functions described above. As flatfish can live in habitats, which differ in hydrology, in chapter 3, we assessed the influence of the abiotic factor salinity on the innate immune parameters applied here in flounder. In a third set of experiments we integrated these innate immune parameters in a multibiomarker study, conducted on flounder in the North Sea. In this study additional chemical and bioaccumulation monitoring as well as established biomarkers of different levels of biological organisation (Figure 1) were applied, which were recommended by the International Council for the Exploration of the Sea (ICES 1996, 1999). The findings of the different monitoring methods are compared. Chapter 4 focusses on the influence of parasites on innate immune parameters, derived from flounder in the

multibiomarker study. Finally, innate immune functions were assessed from dab collected at various locations in the North Sea along a pollution gradient. In this study, biochemical biomarkers of pollution and grossly visible diseases as well as parasites were recorded from the same individuals according to ICES recommendations (ICES 1996, 1999) and the findings of the different measurements were compared. By integrating measurements of innate immune response into biomarker studies we here tried to assess the overall immunomodulatory effect of xenobiotics on fish.

Chapter 2

Measuring some flounder (*Platichthys flesus* L.) innate immune responses to be incorporated in effect biomonitoring concepts

Summary

For an implementation of innate immune responses of flounder (*Platichthys flesus* L.) in an integrated biological effect monitoring concept, leukocytes were isolated from peripheral blood, head kidney and spleen and analysed for their capability of mounting a respiratory burst response upon phorbol ester stimulation. Responding cells were identified by reduced nitro-blue-tetrazolium salt deposits and by di-hydrorhodamine fluorescence in light microscopical and flow cytometric analysis. Responding cells were found in head kidney derived cell suspensions rather than in peripheral blood or spleen. Parallel cytometric and microscopic analysis indicated that responding cells had granulocyte or monocyte morphology, were alpha-naphtyl-esterase or myeloperoxidase positive and in flow cytometry exhibited characteristic forward and side scatter (FSC/SSC) pattern. These cells were present in head kidney derived cell suspensions in proportions of 30-40 % and in peripheral blood and spleen only in proportions of 4-5 %. In order to reduce sampling effort in field studies, leukocyte cell suspensions derived from flounder head kidney could be used in respiratory burst assays without further enrichment protocols. In addition, lysozyme activity could be recorded from flounder plasma in a simple turbidometric assay, which was evaluated by means of a microtiter plate reader. Both assays can be implemented in integrated field studies.

Introduction

Innate immune responses of fish and invertebrates are discussed as promising candidates for biomarkers in an assessment of the impact of pollutants or xenobiotics on aquatic biota (den Besten 1998, Bols *et al.* 2001, Dunier and Siwicki 1993). Innate immune responses protect organisms against infection without depending upon prior exposure to any particular microorganism. This could mean, that it directly reflects interactions of the species with other organisms. In addition, many components of innate immunity appear to be evolutionary conserved (Hoffmann *et al.* 1999, Ulevitch 2000), which could indicate that the sensitivity of an innate immune mechanism to a particular contaminant is similar among species. This would make predictions of impacts of toxicants in the environment easier. Innate responses comprise biochemical and cellular processes, and in laboratory and field studies, many different parameters were monitored (reviewed by Dunier and Siwicki 1993, Bols *et al.* 2001). Promising candidates for use in environmental studies were lysozyme activity and production of reactive oxygen species by phagocytic leukocytes (Bols *et al.* 2001).

Lysozyme is an enzyme that disrupts the cell walls of bacteria (Yano 1996) and is found in various organs and secretions such as mucous layers or serum. In toxicological studies, lysozyme activity was measured by different methods in samples from various origins. In general, lysozyme activity levels were found to be susceptible to xenobiotics, but the results were found to depend on the origin of the sample and the method used for analysis, which makes comparative studies very difficult (reviewed by Bols *et al.* 2001). As serum or plasma can be collected, frozen and evaluated later by an inexpensive assay, lysozyme activity can be incorporated in a monitoring program as a convenient parameter. For a comparison of measurements among studies, standardization of sample processing and analysis is required.

For the assessment of impacts of xenobiotics on cellular responses, the activity of phagocytic leukocytes such as neutrophilic granulocytes or monocytes was monitored in various studies (Secombes *et al.* 1997, Zelikoff 1993, Zelikoff *et al.* 1997). Phagocytosis is a complex process which comprises various steps: migration, adhesion, ingestion of particles, degranulation and respiratory burst by the production of reactive oxygen radicals (ROS, English 1999). Most studies in ecotoxicology focus on the ingestion of particles (phagocytosis) or respiratory burst, which can be measured by means of flow cytometry or in colorimetric assays (Chilmonczyk *et al.* 1997, Secombes 1990). The respiratory burst may be measured in several ways, which again can complicate comparison studies. In addition, cells can be collected from different organs and activated by various stimuli to trigger respiratory burst activity. As the respiratory burst appears to hold promise as a bioindicator for fish health (Rice *et al.* 1996) standardization of experimental protocols among research groups is considered to facilitate its use in environmental studies (Bols *et al.* 2001).

In an attempt to incorporate measurements of innate immune responses in an integrated biological effect monitoring concept in flounder (*Platichthys flesus* L.), leukocytes were isolated from peripheral blood, head kidney and spleen and analysed for the presence of different leukocyte subsets. Cells from these tissues were assessed for their capability of mounting a respiratory burst response upon stimulation by the synthetic agonist phorbol-12-myristate-13-acetate (PMA). The analysis of cellular subsets and respiratory burst response was done using conventional microscopic and colorimetric methods as well as flow cytometric analysis. Flow cytometry-based applications have been used to study various aspects of fish genetics and immunology (Thuvander *et al.* 1992,

Verburg-van Kemenade *et al.* 1994) and only recently were applied in fish toxicology studies (Chilmonczyk *et al.* 1997).

Material and methods

Fish

Flounder (*Platichthys flesus* L.) for this study was collected in the German Bight of the North Sea at a location in the Elbe estuary off Cuxhaven harbour during cruises with the research vessel "Uthörn" of the Alfred Wegener Institute. Sampling campaigns were conducted in April and September 1999 and 2000. Fishing was done with a bottom trawl (opening 1.5 m, mesh width in the cod end 40 mm stretched mesh). The fishing period was limited to 30 min to keep fish stress as low as possible. On board, the fish were sorted out immediately and kept in tanks with permanent seawater flow-through and aeration for up to 6 h until further processing took place. Only macroscopic healthy flounder of the size class 18-25 cm were used for this investigation. Some flounder were dissected on board of the research vessel, blood and tissue samples were taken and processed in the laboratory immediately as described below. Some flounder were brought to the laboratory and kept in 80 l plastic aquaria with recirculated and aerated artificial sea water (Tropic Marin) at 14 °C (±1.5) in the dark. The fish were fed daily ad libitum with frozen shrimps. All fish were acclimatized to laboratory conditions for 2 weeks before they were used for tissue sampling.

Examination procedure

On board of the research vessel, body length and weight were measured from each of the fish and blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-heparin bead (Sarstedt, Germany). The blood was transferred to centrifugation tubes and centrifuged at 2000 x g for 15 min at 4 °C. Then the supernatant plasma was collected and frozen at –80 °C. For flow cytometric analysis, blood was collected in the laboratory by caudal veni puncture into syringes prefilled with heparinised medium (RPMI medium, supplemented with 50 000 IU l^{-1} sodium heparin). Then the fish was killed, dissected and head kidney and spleen were removed and transferred into centrifugation tubes filled with wash medium (RPMI medium supplemented with 10 000 IU l^{-1} sodium heparin; chemicals: Sigma-Aldrich, Germany medium: Biochrom, Germany) and stored at 4 °C for up to 24 h for further processing.

Lysozyme activity

Lysozyme activity of flounder plasma was determined by means of a turbidimetric assay according to Parry *et al.* (1965). A suspension of 0.2 g l^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 5, 10, and 25 µl of flounder plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and then every 30 sec up to 6.5 min at a temperature of 20±2 °C. The decrease in absorbance was used to calculate lysozyme activity. A significant and linear decrease was observed over 4.5 min at a concentration of 25 µl of flounder plasma in 200 µl of *M. lysodeikticus* suspension. As an external standard, hen white egg lysozyme (Sigma-Aldrich, Germany) was used (Hutchinson and Manning 1996). One unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 OD min^{-1}.

Leukocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4 °C. Peripheral blood leukocytes (PBL) were separated from erythrocytes by centrifugation (30 min, 750 x g) over Lymphoprep (Nycomed, Oslo, Norway) as described by Miller and McKinney (1994). Cell suspensions of head kidney leukocytes (HKL) and spleen were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zürich, Switzerland). Isolated HKL, PBL and spleen leukocytes were washed three times with wash medium (10 min, 550 x g) and resuspended in cell culture medium: RPMI-1640 supplemented with 1 % [v/v] carp serum (serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -20 °C until use), 100 000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine (all chemicals: Biochrom, Berlin, Germany). Numbers of viable cells were determined by trypan blue exclusion in a Neubauer haemocytometer.

For density gradient centrifugation of flounder HKL and PBL, a gradient was established by the centrifugation of 20, 40 and 60 % Percoll solutions (Pharmacia, Sweden) at 30 000 x g for 30 min. 3 ml of HKL or PBL suspension were layered on top of the gradient and centrifuged for 30 min, at 750 x g. Fractions were collected separately into centrifugation tubes and washed two times with wash media. The cells were adjusted at a density of 10^6 cells per ml and cytospin preparations were made for

cytological staining and cytochemistry. In addition, samples of these cells were subjected to flow cytometric analysis.

To lyse erythrocytes in cell suspension of spleen or peripheral blood, the cells were exposed to distilled water for 5 s, 10 s, 15 s, 20 s, 25 s and 30 s. Then the cell suspensions were analysed by means of a flow cytometer.

Production of reactive oxygen species by flounder leukocytes

Generation of reactive oxygen species (ROS) by flounder leukocytes was measured by means of the intra-cellular oxidation of fluorescent di-hydrorhodamine (DHR 123) and by the nitro blue tetrazolium salt (NBT) reduction assay. For the DHR-assay, PBL, HKL and spleen cells (10^6 cells ml^{-1}) were incubated for 15 min in cell culture medium with DHR (1 mgl^{-1}) and 0.15 mg l^{-1} phorbol-12-myristate-13-acetate (PMA, Sigma-Aldrich, Germany) or without PMA in flow cytometer tubes in the dark. Morphology and fluorescence characteristics were recorded immediately after incubation, by means of flow cytometry. In addition, live cells were observed with a fluorescence microscope (Zeiss-Axiophot, Fa. Carl Zeiss, Germany), and micrographs were taken on Ilford Pan F 50 film. For the NBT assay, head kidney leukocytes were incubated in 96-well flat-bottom microtiter plates (10^6 cells in a final volume of 175 µl of cell culture medium). All set-ups were made at least in triplicate. Receptor-independent ROS production was induced by adding 0.15 mg l^{-1} PMA. The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the spontaneous ROS generation of cells. After incubation for 2 h at 18 °C, the supernatants were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol. Methanol was removed and the fixed cells were air dried over night and stored in the dark for up to two weeks. The reduced NBT (formazan) was dissolved in 125 µl 2 M KOH and 150 µl DMSO per well (All chemicals: Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm. Additional replicates with cells in NBT were taken for microscopic examination with a photo microscope („Zeiss-Axiophot“, Fa. Carl Zeiss, Germany).

Cytochemistry

Myeloperoxidase staining

Air dried smears of HKL cell suspension were fixed for 30 s in a mixture of 37 % formalin and 95 % ethanol (relation 1:10, respectively), rinsed 2 min under tap water

and air dried in the dark (Kaplow, 1966). Activity of myeloperoxidase was visualized by means of diaminobenzidine (DAB, Fluka-Chemie AG, Switzerland), which in the presence of H_2O_2 is oxidized by the enzyme myeloperoxidase and gives a brown pigment (Lojda *et al.* 1976 modified from Graham and Karnovsky (1966)). Staining of nuclei was done with Harris`-haematoxylin-solution (Merck, Germany).

Nonspecific Esterase activity

Air dried smears from HKL cell suspension were fixed in a formalin saturated atmosphere for 2 min (Lin *et al.* 1998). A modified staining method with α-naphthyl acetate (Sigma-Aldrich, Germany) according to Davis and Ornstein (1959) was used to detect esterase activity (Romeis 1989). According to this method, α-naphthyl acetate in the presence of freshly formed diazonium salt of pararosaniline is enzymatically hydrolysed by the esterase, liberating free naphthol compounds. These then couple with the diazonium salt to give a red coloured deposit at the site of the enzyme. The nuclei were counterstained with Harris` haematoxylin solution (Merck, Germany).

In addition, smears were air dried, fixed with methanol for 3 min and stained with Giemsa (Merck, Germany).

Slides were observed with a photo-microscope („Zeiss-Axiophot“, Zeiss, Germany), and microphotographs were taken using an Ilford, Pan F film.

Flow cytometric analysis of leukocytes

Cell suspension of peripheral blood, head kidney and spleen were analysed with a flow cytometer (FACScan®, Becton Dickinson, Germany, single excitation wavelength of 488 nm) immediately after isolation and after incubation with DHR. Plates with cultured cells were placed on ice (15 min), briefly shaken, then the whole content of each well was transferred to individual flow cytometer tubes and 2 mg l^{-1} propidium iodide (Calbiochem, Bad Soden, Germany) was added to each tube. Forward (FSC) and side scatter (SSC) characteristics of 10 000 events were acquired in linear mode, fluorescence intensities at wavelengths of 530 nm, and 650 nm were acquired at log scale. All flow cytometric data were analysed with the software WinMDI, version 2.8 (Trotter 1998). Cellular debris with low FSC characteristics and propidium iodide-positive, dead cells were excluded from further evaluation.

Statistics

Correlations between leukocyte numbers and ROS readings were calculated with Pearson's product moment correlation or with Spearman's rank correlation. Correlations were considered to be significant at $p<0.05$.

Results

Lysozyme

Lysozyme activity of flounder plasma could be measured by lysis of *Micrococcus lysodeikticus* in an assay adapted to a microplate reader. For a significant and linear decrease of optical density over a period of 4-5 min, 25 µl of flounder serum had to be mixed with 200 µl of *M. lysodeikticus* suspension. In contrast to Hutchinson and Manning (1996), who noted a sharp loss of lysozyme activity in dab serum upon storage at –20 °C, lysozyme activity of flounder plasma was retained after 1 and 4 weeks of storage (data not shown).

Leukocyte typing and ROS-production

Flow cytometric analysis of peripheral blood and spleen leukocytes of flounder yielded 1 main population with small FSC/SSC profiles (Fig. 1 a, b, Region 1, R1). In parallel microscopic evaluation, these cells showed the morphology of lymphocytes (Fig. 2). Cells from the head kidney exhibited a more complex pattern (Fig. 1 c). Three major populations could be differentiated: cells with small FSC/SSC profiles in R1, a cell population with increased FSC/SSC characteristics in R2 and a third population with low SSC values but increased FSC values in R3 (Fig. 1c).

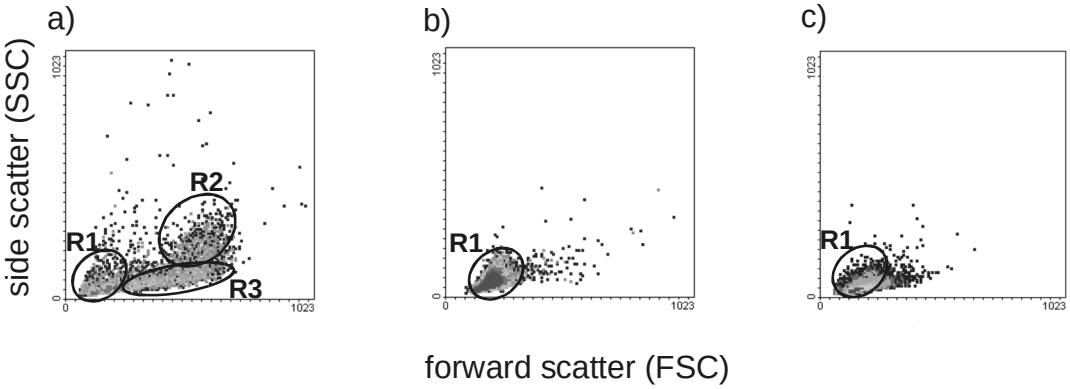
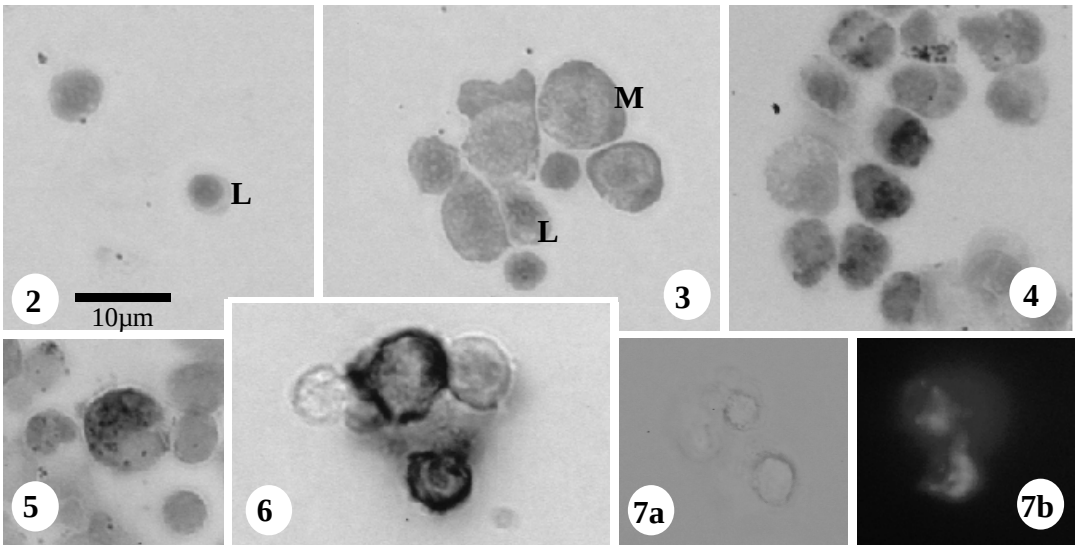


Figure 1: Flow cytometric characteristics of flounder leukocytes from (a) head kidney, (b) peripheral blood and (c) spleen. Note that cell suspensions derived from peripheral blood and spleen contained only 1 cell population (region 1, R1; small cells with low FCS/SSC characteristics), whereas cells from the head kidney had a more complex pattern with cells with increased FSC/SSC characteristics (R 2) and cells with increased cell size (R3).

In microscopical analysis, small lymphocytes with a condensed nucleus, and larger cells with an increased nucleus/ plasma ratio were observed. In Giemsa stained slides, these cells were identified as granulocytes and monocytes (Fig. 3). In addition, granulocyte type cells exhibited a strong myeloperoxidase activity (Fig. 4) and cells with monocyte morphology were positive for α-naphthyl-esterase activity (Fig. 5).

In an attempt to analyse different leukocyte subsets from the head kidney for their ability to produce reactive oxygen species, cells were incubated in the presence of NBT or DHR. Microscopical observation revealed that granular cells had incorporated blue NBT formazan deposits (Fig. 6) and, upon DHR incubation, exhibited a clear green fluorescence (Fig. 7). Flow cytometric analysis (Fig. 8) showed that in HKL suspensions cells from the region R2 responded to PMA stimulation with ROS production. As ROS-production is a functional characteristic of granulocytes and monocytes (Secombes, 1996), it can be concluded, that these cells were found in the R2 region in flounder HKL suspensions.



Figures 2-7: *Platichthys flesus* leukocytes from peripheral blood and head kidney. Fig. 2: Peripheral blood derived lymphocytes. Figs. 3-7: Head kidney derived leukocytes. Fig. 3: lymphocytes (L), monocytes (M) Giemsa stain. Fig. 4: Myeloperoxidase reaction. Fig. 5: α-naphthyl-esterase reaction. Fig. 6: Nitro blue tetrazolium deposits in leukocytes. Fig. 7: Di-hydrorhodamine fluorescence. Fig. 7a: Phase contrast image of 3 leukocytes Fig. 7b: The same cells under UV illumination: 2 cells exhibit a clear fluorescence.

Flow cytometric quantification of cellular subsets revealed that head kidney derived leukocytes consisted of about 30 % cells from R2, while in peripheral blood or spleen, these cells were present in a proportion of 1.5 to 4.5 % only (Table 1). Likewise, in HKL the numbers of ROS producing cells and spectrophotometric NBT readings were significantly higher than in PBL or spleen suspensions (Table 1). In cell suspensions, the number of ROS-producing cells as determined by DHR-fluorescence corresponded with the number of cells found in R 2 ($R=0.982$, $p<0.001$, $n=9$). In addition, the number of DHR-fluorescent cells corresponded to parallel readings using the colorimetric NBT assay ($R=0.719$, $p<0.001$, $n=18$).

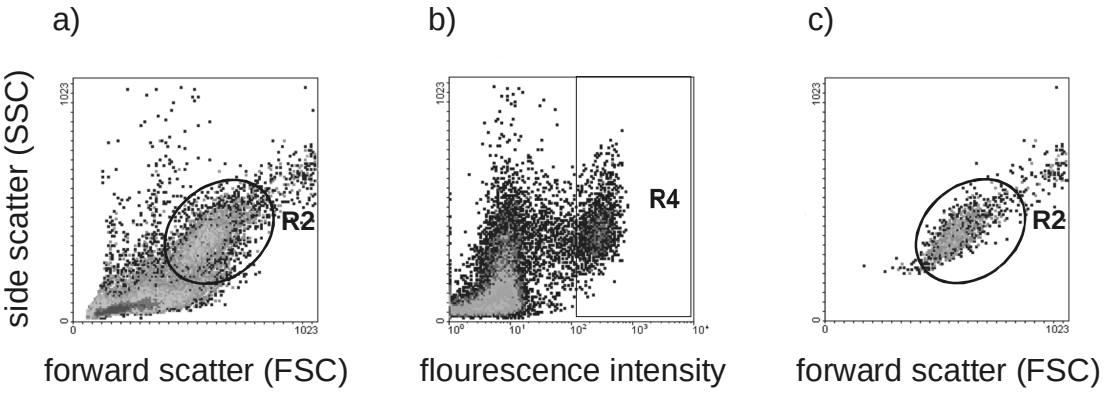


Figure 8: Flow cytometric detection of respiratory burst activity in head kidney derived leukocytes. A: FSC/SSC scatter diagram of a head kidney cell suspension. B: Green fluorescence/SSC scatter diagram of the same cells after incubation with di-hydrorhodamine and stimulated with the phorbol ester PMA. Measurements gated in R4 were considered as fluorescent positive, which indicated respiratory burst activity. C: FSC/SSC profiles of green fluorescence positive cells from the region R4 are characteristic for cells from the region R2 in Fig. 1.

Table 1: *Platichthys flesus*, proportion of leukocyte subsets and ROS production in lymphoid organs. Cells were characterised in flow cytometric analysis by FSC/SSC scatter profiles according to Fig. 1. Cells from region R 1 were characterised by low FSC (cell size) and SSC (cell complexity) profiles, while cells from region R 2 had increased FSC/SSC characteristics (see Fig. 1). The ROS production of corresponding cell suspensions was measured by means of the colorimetric NBT assay and the percentage DHR-fluorescence positive (pos.) cells measured by flow cytometry. Data show mean and standard deviation of measurements from 4 individual flounder. Significant differences in cell distribution and ROS production were found between head kidney and peripheral blood or spleen measurements.

Tissue	% cells in the gate		NBT (O_2^-)	DHR (H_2O_2)
	R 1	R 2	(optical density)	(% pos. cells)
head kidney	52.4(±2.7)	27.8(±3.3)	0.320(±0.109)	41.5(±11.1)
peripheral blood	90.4(±4.3)	4.4(±2.2)	0.143(±0.101)	4.5(±3.3)
spleen	93.9(±2.5)	1.5(±0.6)	0.033(±0.019)	1.5(±1.3)

In an attempt to purify phagocytes from crude head kidney cell suspensions, HKL were centrifuged on continuous gradients prepared from 20, 40, and 60 % Percoll gradients. After centrifugation on a 60 % Percoll gradient, 3 populations were obtained, two major populations at a density of 1.05-1.06 g l^{-1} and a third minor population at a density of 1.08-1.09 g l^{-1}. Flow cytometric and microscopical analysis showed that lymphocyte, granulocyte and monocyte type cells were present in all 3 populations. While population 1 mainly consisted of lymphocytes (50- 60 % of cells in region R1), population 2 was enriched for granulocyte/ monocyte type cells (50- 60 % of cells in region R2, Fig. 9). Population 3 consisted of a ratio of 50- 55 % of cells from R1 and 40- 45 % of cells from R 3 (n=3, Fig. 9). In cell lysis experiments, cells from R 3 were identified as erythrocytes (not shown).

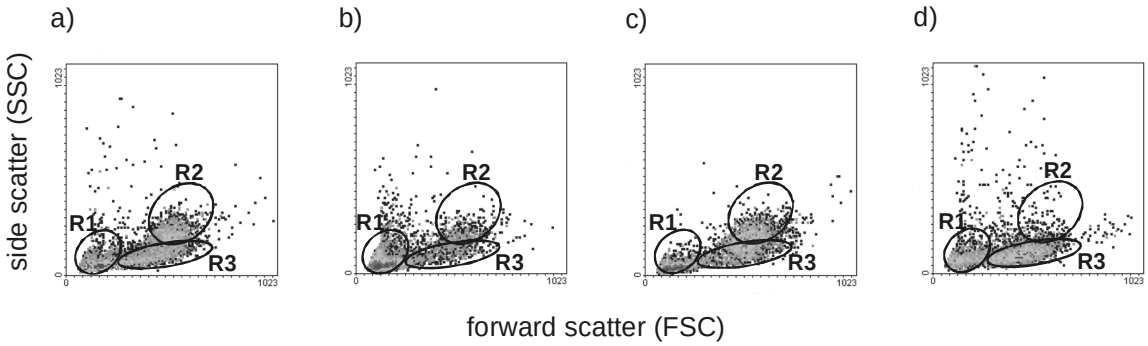


Figure 9: FSC/SSC profiles of head kidney cells after centrifugation on a continuous gradient generated from a 60 % Percoll solution. A: freshly isolated suspension. B: population 1, at a density of 1.05 g l^{-1}, C: population 2 at a density of approx. 1.06 g l^{-1}, population 3 at a density of approx. 1.08 g l^{-1}. Population 3 contained a high number of erythrocytes and was red in colour.

Discussion

European Flounder (*Platichthys flesus* L.) is frequently used in biomonitoring studies conducted in coastal environments of Northern Europe (Grinwis *et al.* 2000, Roose *et al.* 1998, Broeg *et al.* 1999). In these waters, the flounder is widely distributed, lives in close contact to the sediment and is tolerant to changes in salinity which enables the fish to invade estuaries as well. Studies in flounder focus on chemical (Grinwis *et al.* 2000), pathological and parasitological (Broeg *et al.* 1999) analysis but also comprise measurements of innate immune parameters (Boonstra *et al.* 1996, Pulsford *et al.* 1995, Grinwis 1998). Among these, serum lysozyme activity and responses of phagocytic cells were most promising candidates for biomarkers. These parameters were found to be susceptible to xenobiotics and can be measured in high through-put, inexpensive assays (Bols *et al.* 2001).

For an implementation of these parameters in an environmental monitoring study we here analysed the capability of flounder leukocytes for their respiratory burst activity. NBT-formazan deposits as an indicator of intracellular secretion of superoxide anion were observed in leukocytes with a granulocyte or monocyte morphology. These cells also exhibited myeloperoxidase activity, which was found to be characteristic for granulocytes of many fish species (Hine *et al.* 1987, Lehmann *et al.* 1994). In flow cytometric analysis, only cells with increased FSC/SSC pattern exhibited respiratory burst activity. In carp (Verburg-van Kemenade *et al.* 1994) and rainbow trout (Chilmonczyk *et al.* 1997) cells with this FSC/SSC pattern and these functional characteristics were regarded as granulocytes and monocytes. Like in rainbow trout and

carp (Chilmonczyk *et al.* 1997, Scharsack *et al.* 2001), head kidney derived leukocytes of flounder contained increased proportions of cells with granulocyte or monocyte morphology and also showed increased respiratory burst activity. In peripheral blood and spleen, phagocytic cells were present in small numbers, and cell suspensions from these tissues gave only low respiratory burst measurements in response to phorbol ester stimulation. In field studies, collection of peripheral blood leukocytes is less invasive, but due to the low signal obtained, their respiratory burst activity is difficult to measure. In addition, a neutrophil influx into the peripheral blood is observed upon acute stress (Scharsack *et al.* 2001, Chilmonczyk *et al.* 1997) or infection (Scharsack *et al.* 2000), which will cause an increase in respiratory burst activity and thus interfere with pollution mediated effects. In head kidney, stress or infection related effects on proportion and activity of neutrophils can also be noticed when their respiratory burst activity is assessed (Chilmonczyk *et al.* 1997). Upon infection, most prominent effects were seen in basal ROS production (Scharsack *et al.* 2000), which, however is more likely to represent routine metabolic activity of cells than an immune response (Turrens and Boveris 1980). Thus it might be concluded that measurements of basal ROS production or calculation of ROS stimulation indices have no major relevance in pollution monitoring studies.

Enrichment of ROS producing cells from crude head kidney cell suspensions was received by density gradient centrifugation. Crude suspensions contained 30- 40 % of ROS producing cells. After density gradient centrifugation, this proportion increased up to 60 %. In common protocols for isolation of macrophages or granulocytes (Secombes 1990), density gradient centrifugation is followed by adhesion of cells to culture vessels and non adherent cells will be washed away. Attachment to surfaces, however, is a step in the process of phagocytosis (English 1999), which also could be modulated by xenobiotics. In experiments, where the ability of cells to attach to surfaces is impaired by xenobiotics, these cells would be lost during washing procedures, and respiratory burst activity would be measured only from those cells, which retained their ability to attach to surfaces. In result, the signal might be too low, because a significant number of cells would be lost, or it might be too high, because the purification procedure selected for less affected cells. To avoid losses of non-adherent ROS-producing cells, we measured respiratory burst activity of cells in suspension. In order to minimize sampling effort for application in a field study, crude cell suspensions were used. We are aware, that this procedure does not allow an adjustment of respiratory burst measurements

according to phagocyte numbers and might result in high variation of readings. Flow cytometric evaluation of ROS production allowed an identification and quantification of responding cells. In addition to cell function, it allows a simultaneous observation of cell morphology and changes in the proportion of cellular subsets, but it relies on the analysis of live cells (Scheffold and Radbruch 1998). The FSC/SSC characteristics of head kidney derived flounder leukocytes changed upon formalin fixation and storage, which did not allow morphometric analysis of cells at a later date (data not shown). The colorimetric NBT assay has to be performed with live cells as well. Methanol fixed, air dried plates could be stored for later analysis.

In conclusion, respiratory burst active phagocytes could be isolated from the head kidney of flounder in sufficient numbers to give good readings of ROS production in flow cytometric as well as in colorimetric assays. The NBT assay and phorbol-ester stimulation of the cells gave reliable results. In addition, isolation and processing the cells could be implemented in a work schedule for an intergrated program in environmental monitoring.

Chapter 3

The effect of experimental salinity change *in vivo* on some innate immune responses of euryhaline European flounder (*Platichthys flesus* L.)

Summary

A decrease in ambient salinity from 32 to 16 ppm did not result in a redistribution of leukocyte subsets in the head kidney of the euryhaline European flounder (*Platichthys flesus* L.). Phagocyte functions of head kidney derived leukocytes, such a respiratory burst and pinocytosis activity as well as plasma lysozyme levels also were not altered upon the change in salinity. The immune parameters considered here are regarded as promising indicators of chemical contaminant induced variation of piscine immune responses, which could be implemented in pollution monitoring programs. The findings here indicate that these parameters are not sensitive to salinity changes in brackish or estuarine environments.

Introduction

Chemical contaminant exposure has the potential of compromising immune functions that can lead to altered resistance of hosts to pathogens and to increased tumor susceptibility (Dean *et al.* 1986). In the mammalian system, alterations of immune responses and variation of disease resistance are very sensitive indicators, at least in part, of toxic insult to the immune system (Luster and Rosenthal 1993). To identify immune system changes upon exposure to chemicals, a panel of assays was developed and typically is used (Luster *et al.* 1988). These assays originally developed for rodents, have been adapted for use in fish species and are in use for an assessment of the immunotoxicology of different chemical classes in laboratory studies (Zelikoff 1998, Zelikoff *et al.* 2000). In a polluted environment, fish are exposed to a cocktail of largely unknown compounds for a not defined period. For pollution monitoring studies in the marine environment for instance, alteration of innate immune responses such as phagocyte functions or plasma lysozyme activity were considered as most promising candidates for indicators of environmental pollution (Bols *et al.* 2001, den Besten 1998, Dunier and Siwicki 1993).

In international monitoring programmes of biological effects of contaminants in coastal waters and estuaries bottom dwelling marine flatfish species as European flounder (*Platichthys flesus* L.) are frequently used as a sentinel species (ICES 1996, ICES 1999). The flounder is widely distributed in different benthic habitats of the North and Baltic Sea. Due to its euryhaline nature, it migrates into estuaries and is also found in riverine freshwater habitats. While the effect of different ambient salinities on physiology (e.g. Waring *et al.* 1992) and biotransformation of xenobiotics (Schlenk *et*

al. 1996a, 1996b) was evaluated, knowledge about the impact of salinity changes on innate immune response considered as bioindicators for chemical exposure is lacking. Therefore, in this study cellular subsets, respiratory burst and phagocytosis activity of head kidney derived leukocytes of flounder were analysed in response to a decrease in salinity from 32 to 16 ppm over a period of 4 weeks. The aim of the present study was to evaluate the impact of a decreased salinity on the immune parameters assessed in a combined pollution monitoring program on flounder to mimic the situation of a brackish environment as it is found in estuaries or the Baltic Sea.

Material and methods

Fish

Flounder (*Platichthys flesus* L.) for this study was collected in the German Bight of the North Sea at a location at Tiefe Rinne near Helgoland during cruises with the research vessel "Uthörn" of the Alfred Wegener Institute. Sampling campaigns were conducted in April and May 1999. Fishing was done with a bottom trawl (opening 1.5 m, mesh width in the cod end 40 mm stretched mesh). The fishing period was limited to 30 min to keep fish stress as low as possible. Flounder were brought to the laboratory and kept at 15 °C with permanent sea water flow-through and aeration. The fish were fed daily ad libitum with frozen mussels. All fish were acclimatized to laboratory conditions for 3 weeks before they were used for the experiment. Flounder (n=100) were randomly divided in two groups. For one group the salinity was artificially decreased from 32 ppm to 16 ppm within 24 h, by continuous dilution with freshwater, while the other group remained at 32 ppm during the whole experiment.

Examination procedure

At days 1, 3, 8, 15 and 29 after salinity change, 5-10 flounder individuals were collected from both groups. Body length and weight were measured from each individual and blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-heparin bead (Sarstedt, Germany). The blood was transferred to centrifugation tubes and centrifuged at 2000 x g for 15 min at 4 °C. Then the supernatant plasma was collected and frozen at –20 °C. The fish were killed, dissected and the head kidney was removed and transferred into centrifugation tubes filled with wash medium (RPMI medium supplemented with 10 000 IU l^{-1} sodium heparin; chemicals: Sigma-Aldrich, Germany, medium: Biochrom, Germany) and stored at 4 °C for up to 24 h for further processing.

From the morphological measurements, a whole body condition factor (CF) was determined for each individual according to the formula:

$$CF = (\text{body weight in g} / \text{body length in cm}^3) \times 100$$

and used as an allometric index for overall health (Busacker *et al.* 1990). Hepatosomatic index (HSI) was calculated according to Htun-han (1978) as follows:

$$HSI = (\text{liver weight in g} / \text{whole body weight in g}) \times 100$$

Lysozyme activity

Lysozyme activity of flounder plasma was determined by means of a turbidimetric assay according to Parry *et al.* (1965). A suspension of 0.2 g l^{-1} Micrococcus lysodeikticus (Sigma-Aldrich, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl of flounder plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and then after 4.5 min at a temperature of 20±2 °C. The decrease in absorbance was used to calculate lysozyme activity. As an external standard, hen white egg lysozyme (Sigma-Aldrich, Germany) was used (Hutchinson and Manning 1996). One unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001 OD min^{-1}.

Leukocyte isolation

Leukocyte isolation was done as described previously (Chapter 2). Briefly, cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zürich, Switzerland). Isolated HKL were washed three times with wash medium (10 min, 550 x g) and resuspended in cell culture medium: RPMI-1640 supplemented with 1% [v/v] carp serum (serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -20 °C until use), 100 000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine (all chemicals: Biochrom, Berlin, Germany). Numbers of viable cells in suspension were determined by means of flow cytometry.

Production of reactive oxygen species by head kidney leukocytes

Generation of reactive oxygen species (ROS) by head kidney leukocytes (HKL) was measured by means of the nitro blue tetrazolium salt (NBT, Sigma-Aldrich, Germany)

reduction assay after cell isolation, as described above. Cells were incubated in 96-well flat-bottom microtiter plates (10^6 cells in a final volume of 175 µl of the respective culture medium). All set-ups were made at least in triplicate. Receptor-independent ROS production was induced by adding 0.15 mg l^{-1} phorbolmyristate acetate (PMA, Sigma-Aldrich, Germany). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the base line ROS generation of the cells. After incubation for 2 h at 18°C, the supernatants were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol. Methanol was removed and the fixed cells were air dried over night and stored in the dark for up to two weeks. The reduced NBT (formazan) was dissolved in 125 µl 2 M KOH and 150 µl DMSO per well. The optical densities were recorded with a spectrophotometer at 650 nm.

Endocytosis activity of head kidney phagocytes

Endocytosis activity of head kidney phagocytes was measured by means of neutral red retention of isolated head kidney cells as described by (Mathews *et al*. 1990). This assay was adapted to microtiter plates. Briefly, 10^6 cells were incubated in a final volume of 175 µl culture medium for 2.5 h at 18 °C with 10 mg l^{-1} neutral red (NR, Sigma-Aldrich, Germany). All set-ups were made at least in triplicate. After incubation each well was washed two times with 125 µl phosphate buffered saline (PBS). After removing the PBS, the cells were air dried over night and frozen at -20 °C for up to two weeks. For spectrophotometric readings the cells were lysed with 100 µl acid ethanol (3 % HCl in 95 % Ethanol) and mixed with 100 µl PBS. The optical densities were recorded at 492 nm.

Flow cytometric analysis of leukocytes

Cell suspension of head kidney were analysed with a flow cytometer (FACScan®, Becton Dickinson, Germany, single excitation wavelength of 488 nm) immediately after isolation. They were transferred to individual flow cytometery tubes and 2 mg l^{-1} propidium iodide (Calbiochem, Bad Soden, Germany) was added to each tube. Forward (FSC) and side scatter (SSC) characteristics of 10 000 events were acquired in linear mode, fluorescence intensities at wave lengths of 530 nm, and 650 nm were acquired at log scale. The number of viable cells was calculated by adding standard cells to each tube (assay modified after Pechold *et al.* 1994). Standard cells, paraformaldehyde-fixed

and FITC labelled bovine mononuclear cells (Schuberth *et al.* 1992, Hendricks *et al.* 2000) were used for quantification of non-labelled vital test cells (viable HKL). All flow cytometric data were analysed with the software WinMDI, version 2.8 (Trotter 1998). Cellular debris with low FSC characteristics was excluded from further evaluation. Standard cells (propidium iodide-positive, FITC positive) could be easily discriminated from viable cells (propidium iodide-negative, FITC negative). Total numbers of cells were calculated according to:

N [vital cells] = Events [vital cells] x Number [standard cell] / events [standard cell]

Different leukocyte subsets were identified according their characteristic forward and side scatter values (FSC/SSC profiles; chapter 2).

Statistics

Normality of data was tested with the Kolmogorov-Smirnow test. Differences between the two groups were analysed by Student's t-test or Mann-Whitney's rank sum test. Differences between the groups were considered to be statistically significant at $p<0.05$ using SigmaStat software package (V2.03, SPSS Science Inc.). Correlation coefficients were calculated with the parametric Pearson's Product Moment Correlation or with the non-parametric Spearman Rank Correlation.

Results and conclusions

Flow cytometric analysis of head kidney derived cell suspensions revealed three major leukocyte subpopulations, which were characterised by different forward- and sideward scatter (FSC/SSC) pattern (Fig. 1): cells with small FSC/SSC profiles (R1 in Fig 1, a cell population with increased FSC/SSC characteristics in region 2 (R2, in Fig 1) and a third population with increased FSC values but small SSC values (R3 in Fig 1).

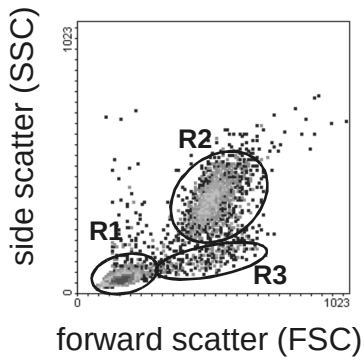


Figure 1: Flounder, *Platichthys flesus*, flow cytometric characteristics of head kidney leukocytes.

The reduction of water osmolarity from 32 ppm to 16 ppm was not accompanied with a change in the proportions of head kidney derived leukocyte subsets (Table 1). An effect of salinity change could not be observed at individual sampling dates as well as at the end of the observation period, after 29 days of exposure. When respiratory burst and endocytosis activity of head kidney cells were observed, enhanced readings were obtained in assays with cells from flounder subjected to salinity change. Differences between the treatment groups could be seen in basal and PMA stimulated ROS production at day 3 of salinity change (Fig. 2). The endocytosis activity showed high individual variations among flounder from both treatment groups, which did not allow to discern differences between the groups (Fig. 3).

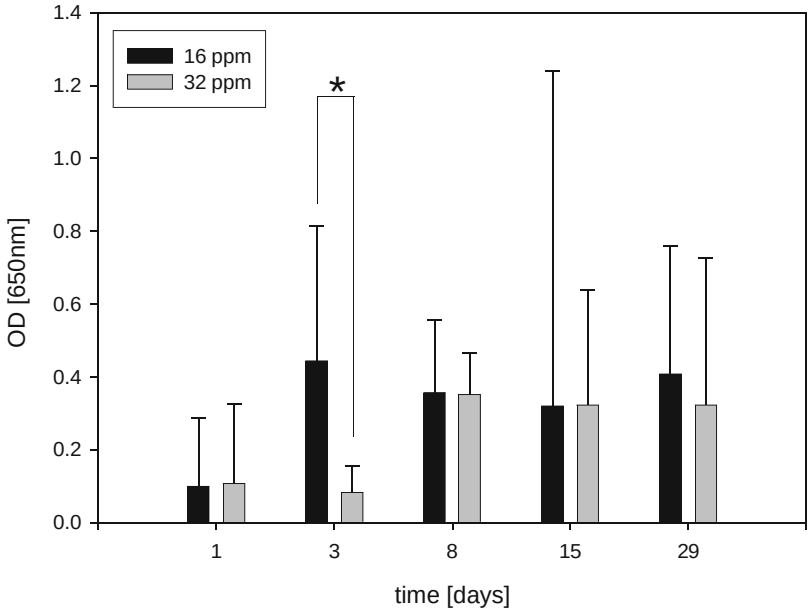


Figure 2a: Basal respiratory burst activity of flounder head kidney leukocytes. Indicated is (*): significant difference between individuals at 16 and 32 ppm salinity

In chapter 2, cells with increased FSC/SSC characteristics from the region R2 (Fig. 1) were observed to generate reactive oxygen species, and respiratory burst readings obtained by the NBT reduction assay were correlated to the number of R2 cells (Chapter 2). In the present investigation, this could be confirmed:

The number of R2 cells present in HKL suspension was positively correlated with the basal ($R=0.41$, $p<0.001$, $n=79$), as well as PMA stimulated ROS production ($R=0.42$, $p<0.001$, $n=79$); and with the endocytosis activity ($r=0.24$, $p<0.05$, $n=79$) from the same individuals.

Table 2: Proportion of headkidney leukocyte populations in flounder after experimental salinity change. Cell populations among head kidney leukocytes were identified flow cytometrically based on their specific forward and side scatter characteristics (chapter 2). Values are means ± SD. Number of fish is denoted in brackets. There are no significant differences between exposed and control groups

Salinity	Day	% cells in R1	% cells in R2	% cells in R3
32 ppm	1	27 ± 7 (10)	32 ± 6 (10)	30 ± 7 (10)
	3	29 ± 7 (10)	28 ± 5 (10)	29 ± 6 (10)
	8	33 ± 9 (5)	29 ± 8 (5)	25 ± 7 (5)
	15	24 ± 7 (5)	37 ± 4 (5)	25 ± 8 (5)
	29	38 ± 6 (5)	27 ± 3 (5)	24 ± 4 (5)
16 ppm	1	32 ± 4 (10)	30 ± 3 (10)	25 ± 4 (10)
	3	35 ± 11 (10)	29 ± 7 (10)	24 ± 6 (10)
	8	35 ± 7 (8)	28 ± 7 (8)	24 ± 6 (8)
	15	27 ± 6 (8)	33 ± 7 (8)	26 ± 10 (8)
	29	31 ± 8 (8)	28 ± 5 (8)	28 ± 7 (8)

The plasma lysozyme activity in flounder did not show alterations which could be related to the salinity change (Fig. 4), but they were elevated day 3 and 5 post salinity change compared to day 29.

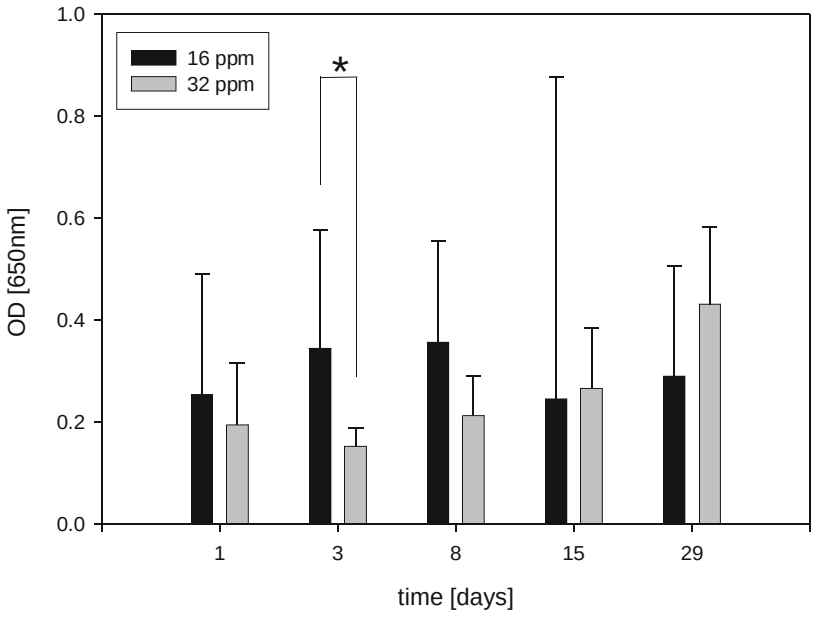


Fig. 2b: PMA stimulated ROS production of flounder head kidney leukocytes. Indicated is (*): significant difference between individuals at 16 and 32 ppm salinity

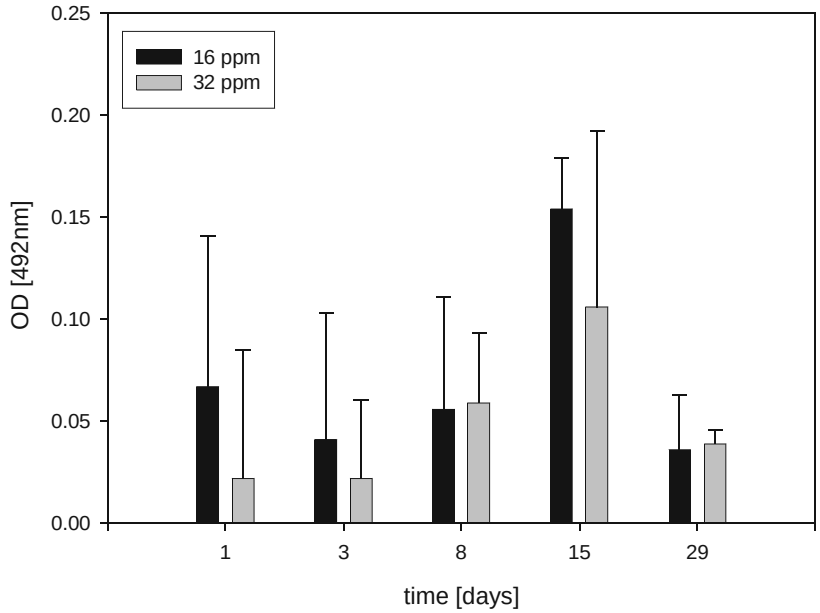


Figure 3: Endocytosis activity of flounder HKL.

The immune measured here (plasma lysozyme activity, basal and PMA activated respiratory burst of head kidney leukocytes and pinocytosis activity of HKL) were not influenced by fish related parameters such as sex, length, weight, condition factor or hepatosomatic index of the flounder examined (data not shown).

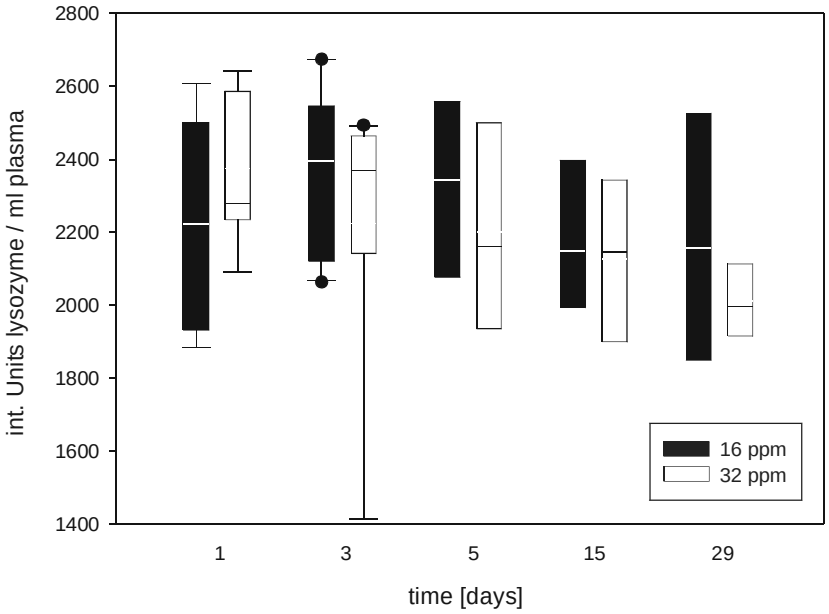


Figure 4: Plasma lysozyme activity in flounder after a 50 % decrease in ambient salinity.

The overall effect of changes in ambient salinity in flounder was a higher energy expenditure associated with increased branchial Na^+, K^+ ATPase activity (Sampaino *et al.* 2002). This apparently did not induce a re-distribution of leukocytes subsets in the head kidney, or an alteration of other innate immune parameters measured here. After a period of acclimatisation for 8 days, no difference could be seen in plasma lysozyme content, endocytosis or respiratory burst activity of HKL between the two treatment groups. During the course of the experiment, at day 3 post salinity decrease the basal and PMA triggered production of ROS by HKL was increased in the group subjected to the salinity drop compared to individuals kept at stable salinity. This however, was not associated with a change in the proportion of ROS producing cells, as indicated by flow cytometric analysis. Other studies on sea bream (*Sparus aurata* L.) showed a decrease of respiratory burst active cells in the head kidney upon handling or anaesthesia induced stress (Ortuno *et al.* 2002). Most likely, these cells migrated from the head kidney into the peripheral blood, as observed in several fish species upon acute stress (Scharsack *et al.* 2001, Ortuno *et al.* 2001, Chilmonczyk *et al.* 1997). Stressed induced alterations in cell functions and humoral components were described by various authors for different stressors (Yin *et al.* 1995, Ortuno *et al.* 2001, 2002). From our findings we conclude that the decrease in salinity from 32 to 16 ppm did not significantly impact flounder innate immune responses. In exposure studies on flounder, the overall effect of reduced ambient salinity was a higher energy expenditure associated with increased branchial Na^+, K^+-ATPase activity (Sampaio *et al.* 2002).

In conclusion, a reduction to ambient salinity by 50 % did not result in an alteration of cellular subsets of head kidney leukocytes and non specific immune functions in euryhaline flounder. Thus in a monitoring program, ambient salinity might not significantly impact measurements of the immune parameters considered here.

Chapter 4

The use of innate immune responses as biomarkers in a program of integrated biological effects monitoring on flounder (*Platichthys flesus* L.) from the southern North Sea

Summary

Immunological biomarkers that reflect the effects of exposure to environmental contaminants in coastal marine habitats were sought in European flounder (*Platichthys flesus* L.) from 5 locations in the German Bight with different anthropogenic impact. During a 2 years period of sampling, innate immune responses were monitored from a total of 331 individual flounder of a body length of 18 to 25 cm. From the fish, plasma lysozyme, phagocytosis and respiratory burst activity of head kidney leukocytes were analysed and implemented as part of an integrated biological effects monitoring program. As at some locations the measurements of the parameters applied here varied within wide ranges, spatial differences not always could be established, but some general trends could be drawn: Plasma lysozyme activity was decreased in flounder contaminated with DDT adducts and some PCBs, while cellular functions such as phagocytosis and respiratory burst were stimulated by some chlorinated hydrocarbons. Correlation analysis also revealed not only connections between the parameters applied here and some contaminants but also with some biochemical parameters used as biomarkers in pollution monitoring: In flounder with decreased integrity of lysosomal membranes of hepatocytes, immune functions also were impaired, and plasma lysozyme as well as phagocytosis activity of head kidney cells were impaired when the activity of cytochrome P450 1A was induced. The data presented here indicate that innate immune responses may be useful parameters to monitor cellular functions in a battery of biomarkers of different levels of biological organisation.

Introduction

In the last few decades, a possible influence of environmental pollution on the aquatic environment has gained considerable interest. Fish have become a favourable subject for research in this area, because temperature changes, habitat and water quality deterioration as well as aquatic pollution adversely affect fish health, which may result in mortalities and population decline. Among various biochemical, cellular and physiological systems, certain innate immune responses are considered as suitable biomarkers for monitoring biological effects of pollution (reviewed by Dunier and Siwicki 1993, Wester *et al.* 1994, Bols *et al.* 2001). Impairment of immune functions, which protect fish against invading pathogens, can lead to harmful consequences on individual level, as disease outbreak followed by death of the individual, and on the ecosystem level, as population reduces followed by the change of the entire ecosystem.

For fish populations, a link between environmental pollution and diseases has long been expected (Sinderman 1979, Sniezko 1974) and from studies carried out under defined conditions in laboratory, modulating influences of xenobiotics on immune responses of fishes were concluded (for review see Dunier and Siwicki 1993, Bols *et al.* 2001). Understanding the effect of toxicants on fish innate immunity supports the larger ecotoxicological goal of comprehending the actions of ecotoxicants on fish populations (Bols *et al.* 2001).

Given that the non-specific immune system of fishes constitutes their first line of defence against pathogens (Ingram 1980), cellular and humoral reactions of the innate immune system are of great importance. They are unspecific but effective against pathogens, which try to invade the host. This leads on cellular level to activation and infiltration of one specialized type of leukocytes into the tissue, where invasion took place. These leukocyte subpopulation, which consists of granulocytes and macrophages, is able to ingest pathogens via endocytosis and to kill them intra- or extracellular by a process known as the respiratory burst (Halliwell and Gutteridge 1999). In fish it has been shown that this cell functions can be modulated by xenobiotics and suggested as most promising attempt for the use as an indicator of health (Bols *et al.* 2001). One important humoral component in the innate immune system is lysozyme (E.C.3.2.1.17), which attacks the peptidoglycan layer in the cell wall of predominantly Gram-positive bacteria, and to some extent, also Gram-negative bacteria. Lysozyme is localized in the lysosomes of neutrophils and macrophages and is released into the blood from these cells (Murray and Fletcher 1976). From previous studies it is known that lysozyme activity in fish blood is sensitive to environmental contaminants (Bols *et al.* 2001).

Activity of phagocytic cells, such as endocytosis or respiratory burst and plasma lysozyme levels do not depend on a previous stimulation with a particular antigen. In addition, these responses can be measured in simple and inexpensive spectrophotometer based assay, which make them suitable for field studies.

In the present study we implemented measurements of innate immune responses of fish in an integrated biological effect monitoring program on European flounder (*Platichthys flesus* L.) in the North Sea. The flounder is widely distributed in different habitats of the North and Baltic Sea. Like other marine flatfishes it lives in close contact to the sediment and feeds on various benthic organisms. Thus marine flatfish species are frequently used as a sentinel species in international monitoring programmes of

biological effects of contaminants in coastal waters and estuaries (ICES 1996, ICES 1999).

The data reported here are part of a monitoring program, which was conducted on flounder collected at several locations in the German Bight and which included the analysis of biochemical, pathological and parasitological parameters of the same individual in addition to the measurement of some innate immune responses. The study was supplemented with chemical analysis of chlorinated hydrocarbon residues in the muscle of the same individual fish used for an assessment of biological effects. In addition sediments and invertebrates (*Mytilis edulis*) were collected from the locations where the fish sampling took place. In the chapter here, findings of the immunological analysis were compared to chemical, biochemical and histochemical data for a more complete understanding of pollution impact on fish physiology.

Material and methods

Sampling

Four sampling campaigns in the North Sea were conducted in April and September 1999 and 2000. During these cruises with the research vessel "Uthörn" of the Alfred Wegener Institute, a total of 331 flounder (*Platichthys flesus* L.) were caught at five different locations (Elbe estuary, Inner Eider estuary, Outer Eider, Spiekeroog and Tiefe Rinne near Helgoland (table 1). The stations were determined by their geographical positions (Broeg *et al.*, in press). Fishing was conducted with a bottom trawl (opening 1.5 m, mesh width in the cod end 40 mm stretched mesh). Fishing period was limited to 30 min to keep fish stress as low as possible. Fish were sorted out immediately and kept in tanks with permanent seawater flow-through and aeration for up to 6 h until further processing took place. Only macroscopic healthy flounder of the size class 18-25 cm were used for this investigation. A maximum of twenty fish per site and campaign were collected and prepared for analysis.

Examination procedure

On board of the research vessel, body length and weight from each were fish were measured, and macroscopic visible ectoparasites were collected. Blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-heparin bead (Sarstedt, Germany). From the blood, the haematocrit was determined according to standard procedures (Houston 1990). The remaining blood then was transferred to centrifugation

tubes, centrifuged at 2000 x g for 15 min at 4 °C, and the supernatant plasma was collected and frozen at –80 °C. Then the fish was killed, dissected and the head kidney was removed and transferred into a centrifugation tube filled with wash medium (RPMI medium supplemented with 10 000 IU l^{-1} sodium heparin, medium: Biochrom, Berlin, Germany, heparin: Sigma-Aldrich, Germany) and stored at 4 °C for up to 24 h for further processing. In addition liver, kidney, intestine, gills and muscle samples were collected for parasitological, physiological and biochemical research as well as residual analysis. This was conducted by cooperating working groups from the Alfred Wegener Foundation in Bremerhaven, the Technical University Berlin and the School for Veterinary Medicine in Hannover. The results of these are reported in different publications (Dizer *et al.* submitted, Schmidt *et al.* submitted). From the morphological measurements, a whole body condition factor (CF) was determined for each fish according to the formula:

$$CF = (\text{body weight in g} / \text{body length in cm}^3) \times 100$$

and used as an allometric index for overall health (Busacker *et al.*, 1990).

Table 1: Summary of the sampling program on flounder (*Platichthys flesus*) for analysis of some innate immune parameter in 1999 to 2000. All parameter were measured from same individual.
Fish: number of fish caught; *Hem*: haematocrit; *Lys*: plasma lysozyme activity, *Pin*: pinocytosis; *ROS*: basal reactive oxygen production; *ROS PMA*: production upon stimulation with phorbol-12-myristate-13-acetate (PMA) by head kidney leukocytes

Campaign/ year	Site	Fish	Hem	Pin	ROS	ROS PMA	Lys
Spring 1999	Elbe	20	20	15	16	16	20
	Spiekeroog	9	9	8	9	9	9
	Helgoland	20	20	20	20	20	20
	Inner Eider	15	14	14	13	14	15
	Outer Eider	19	18	18	19	19	17
Autumn 1999	Elbe	20	20	14	20	20	20
	Spiekeroog	20	19	19	20	20	19
	Helgoland	20	19	20	20	20	18
	Outer Eider	20	19	19	20	20	19
Spring 2000	Elbe	20	20	17	19	19	20
	Spiekeroog	9	9	8	9	9	9
	Helgoland	20	20	16	18	18	20
	Inner Eider	18	18	11	15	15	18
	Outer Eider	20	20	9	17	17	20
Autumn 2000	Elbe	20	20	20	20	20	20
	Spiekeroog	20	20	7	7	7	20
	Helgoland	20	20	20	20	20	20
	Outer Eider	20	20	20	20	20	20

Lysozyme assay

Lysozyme activity of flounder plasma was determined by means of a turbidimetric assay according to Parry *et al.* (1965). A suspension of 0.2 g l^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl of flounder plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and after 4.5 min at a temperature of 20±2 °C. The decrease of absorbance was used to calculate lysozyme activity. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001/min. Hen white egg lysozyme (Sigma-Aldrich, Germany) was used as external standard as described by Hutchinson and Manning (1996).

Leukocyte isolation

Leukocyte isolation was done as described previously (chapter 1). Briefly, cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zurich, Switzerland). Isolated HKL were washed three times with wash medium at 550 x g for 10 min and resuspended in cell culture medium (RPMI-1640 supplemented with 100 000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine and 1 % [v/v] carp serum (chemicals: Biochrom, Berlin, Germany; carp serum: serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -20 °C until use). Numbers of viable cells were determined by trypan blue exclusion in a Neubauer haemocytometer.

Production of reactive oxygen species by head kidney leukocytes

Generation of reactive oxygen species (ROS) by head kidney leukocytes (HKL) was measured by means of the nitro blue tetrazolium salt (NBT) reduction assay after cell isolation, as described earlier (chapter 2). Briefly, cells were incubated in 96-well flat-bottom microtiter plates (10^6 cells in a final volume of 175 µl of cell culture medium) in triplicate and their ROS production was induced by adding 0.15 mg l^{-1} phorbolmyristate acetate (PMA). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the spontaneous ROS generation of cells. After incubation for 2 h at 18 °C, the supernatants were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol.

Methanol was removed and the fixed cells were air dried over night and stored in the dark for up to two weeks. The reduced NBT (formazan) was dissolved in 125 µl of 2 M KOH and 150 µl DMSO per well (All chemicals: Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm. From the measurements, a ROS stimulation index was calculated as followed:

SI= (PMA triggered ROS [OD]) / (unstimulated ROS [OD])

Endocytosis activity of head kidney phagocytes

Endocytosis activity of head kidney phagocytes was measured by means of neutral red retention of isolated head kidney cells as described by (Mathews *et al.* 1990). This assay was adapted to microtiter plates. Briefly, 10^6 cells were incubated in a final volume of 175 µl culture medium for 2.5 h at 18 °C with 10 mg l^{-1} neutral red (NR, Sigma-Aldrich, Germany). All set-ups were made at least in triplicate. After incubation each well was washed two times with 125 µl phosphate buffered saline (PBS). After removing the PBS, the cells were air dried over night and frozen at -20 °C for up to two weeks. For spectrophotometric readings the cells were lysed with 100 µl acid ethanol (3 % HCl in 95 % Ethanol) and mixed with 100 µl PBS. The optical densities were recorded at 492 nm.

Analytical chemistry

Residue analysis was conducted by a commercial laboratory that had undergone an intercalibration exercise (Labor für Fischgesundheit, Professor Harz, Bremerhaven). From the fish, ten muscle tissue samples per location were analysed for the contents of standard chlorinated hydrocarbons and heavy metals (Hg, Pb, Cd, Cu). In addition, samples from the sediment at all locations and samples from *Mytilus edulis* were taken from the inner Eider Estuary, Elbe Estuary and Helgoland and analysed for the same parameters. The methods and results of this work are presented by Dizer *et al.* submitted.

Statistics

Normality of the data was tested with the Kolmogorov-Smirnow test. To determine the significance between groups, data were compared by Student's t-test, Mann-Whitney's rank sum test, or by Kruskal-Wallis ANOVA and subsequent multiple comparison by means of the Student-Newman-Keuls method at a probability of error $p<0.05$.

Correlations between data sets were tested by Pearson's product moment correlation test or by Spearman's rank correlation test. Correlations were considered as significant at a probability of error $p<0.05$.

Results

Haematocrit

The haematocrit of flounder blood was measured as a simple, non specific indicator for overall health (Blaxhall 1972, Anderson 1990). In the sample, measurements varied from 18-30 % and had a mean of 23 %. Male founder had a haematocrit of 24 (19–30) %, slightly higher than female with 22.5 (18-26) %. In spring, a much wider variation of haematocrits was observed at all locations when compared to autumn samples. Differences between sample sites, however, could not be discerned. This indicates that major differences in health aberrations such as infections with micro organisms or nutritional deficiencies did not occur between the locations of the study (Blaxhall 1972).

Table 2: Summary of immune parameter of flounder (*Platichthys flesus*) collected from selected locations in the German Bight during 4 sampling campaigns during spring and autumn of 1999 and 2000.

Parameter	Measurements[mean (range)]				
	summary	male	female	spring	autumn
Haematokrit [Vol %]	23 (18-30)	24 (19-30)	22.5 (18-26)	23 (19-30)	23 (18-27)
Plasma lysozyme [Units ml^{-1}]	1090 (606-1816)	1139 (600-1833)	996 (628-1767)	975 (484-1618)	1189 (743-1882)
Endocytosis [OD]	0.046 (0.009-0.138)	0.042 (0.009-0.016)	0.047 (0.008-0.146)	0.057 (0.014-0.153)	0.023 (0.007-0.092)
basal ROS [OD]	0.087 (0.025-0.499)	0.102 (0.029-0.603)	0.078 (0.025-0.489)	0.053 (0.024-0.367)	0.150 (0.036-0.618)
PMA activated ROS [OD]	0.322 (0.095-0.825)	0.331 (0.103-0.915)	0.317 (0.079-0.694)	0.335 (0.070-0.915)	0.319 (0.166-0.683)

Plasma lysozyme activity

With plasma lysozyme activity, no dependence on the sex of analysed flounder could be observed. It increased however with increasing size and weight of the fish with R=0.15, $p< 0.006$ in the size range examined here. Flounder collected in autumn had a lysozyme activity of 1189 (743-1882) units ml^{-1}, significantly ($p< 0.001$) higher than flounder collected in April with 975 (484-1618) units ml^{-1}. These seasonal differences were significant at the Elbe and Helgoland locations (Fig. 1) and were not seen at Spiekeroog and Outer Eider. Site related differences were found in both spring and autumn samplings between flounder from the Elbe and Helgoland location (Fig. 1). In spring flounder collected at Elbe and Inner Eider had significantly lower plasma lysozyme activity compared to flounder from Spiekeroog and Helgoland (Fig. 1).

When muscle residues of flounder were compared to plasma lysozyme activity of the same individual, a positive correlation was found to residues of γ-HCH. The plasma lysozyme activity of flounder was negatively correlated with β-HCH, o,p'-DDD, PCB 101 and PCB 118 residues in the muscle of the same individual (table 4).

In addition, plasma lysozyme activity was correlated to PMA stimulated ROS production of HKL, lysosomal stability and activity of macrophage aggregations in the liver from the same individual. In individuals with increased induction of EROD activity in liver cells, the plasma lysozyme activity was decreased (table 5).

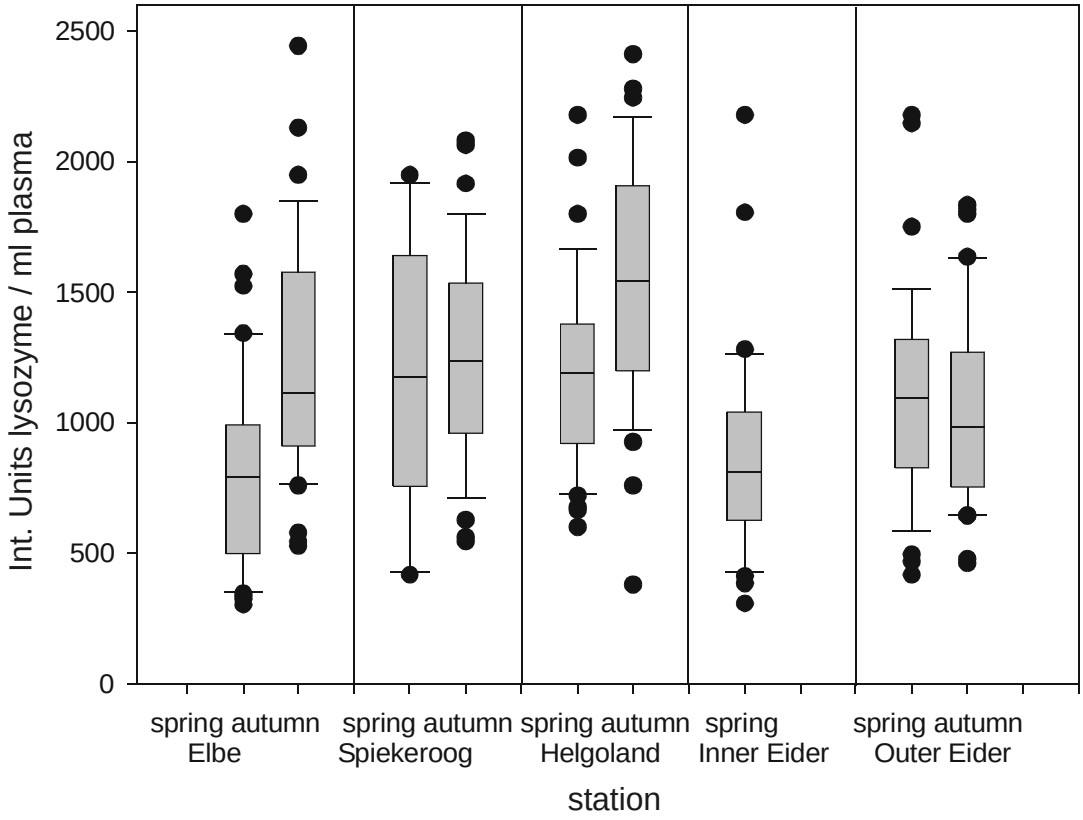


Figure 1: Plasma lysozyme activity in flounder (*Platichthys flesus*) collected at 5 different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. n: see table 1.
Significant differences in spring: Spiekeroog vs. Elbe ($p=0.003$) and Inner Eider ($p=0.023$); Helgoland vs. Elbe ($p<0.001$) and Inner Eider ($p=0.003$); Outer Eider vs. Elbe ($p=0.023$). In autumn campaigns: Helgoland vs. Outer Eider ($p<0.001$), Elbe ($p=0.007$) and Spiekeroog ($p=0.009$).
Key for box plots: boundary of the boxes: 25th and 75th percentile; line in the box: median; whiskers: 10th and 90th percentiles; dots: outliers

While no correlation of plasma lysozyme activity was observed with the concentration of chlorinated hydrocarbons or heavy metals in the sediment, negative correlation of plasma lysozyme activity was seen with a standardized contamination load in *Mytilus edulis* collected from the sediments (Fig. 2, Spearman's Rank Correlation, $p<0.05$). It has to be noted, that the n value for this correlation was very low (n=10), but nevertheless the contamination load of *Mytilus* might reflect the contamination of the food used by flounder at a particular location.

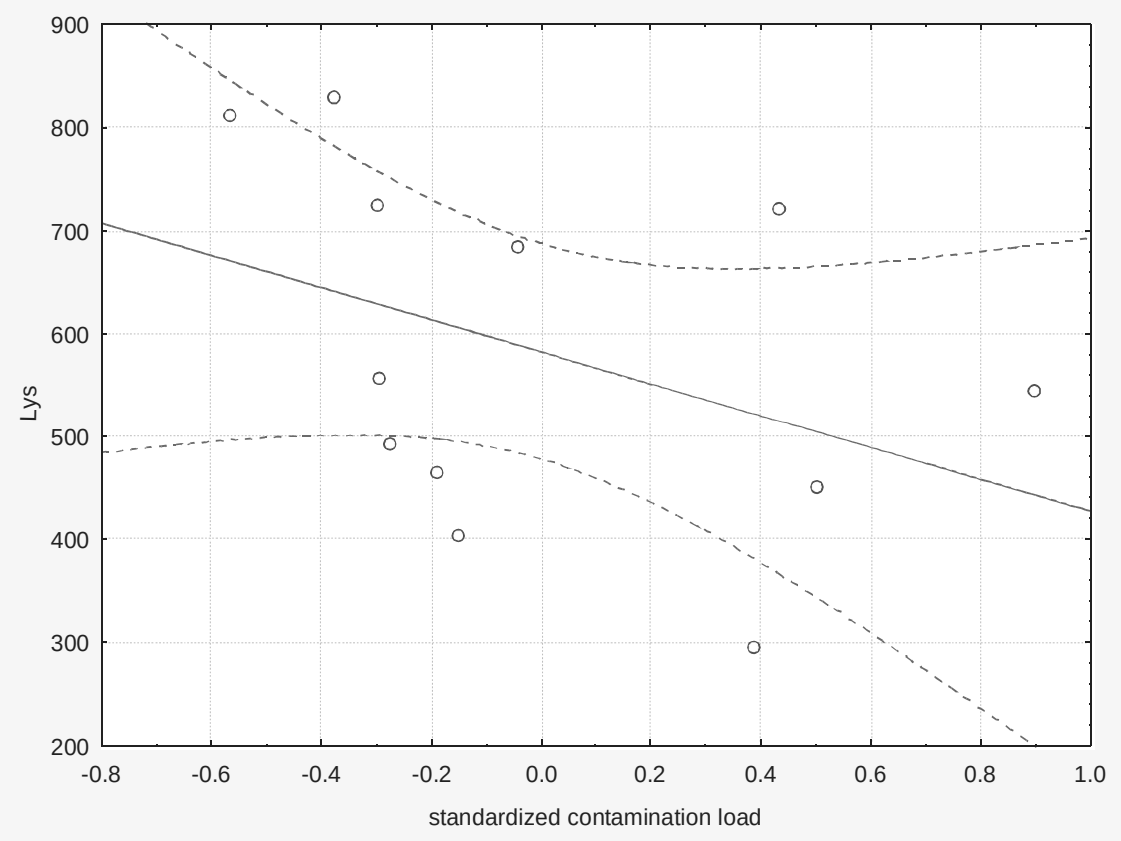


Figure 2: Correlation between mean plasma lysozyme activity in flounder (*Platichthys flesus*) and mean standardized contamination load in *Mytilus edulis* collected at the Helgoland, Spiekeroog and Eider sites. The contamination load of mussel tissue was calculated from standardized mussel residues (data from Dizer *et al.* submitted). Spearman's Correlation on Ranks coefficient R= -0.58, $p<0.05$, dotted lines: 95% confidence interval; *Lys*: int. Units lysozyme/ml plasma

Cellular responses

Endocytosis by head kidney phagocytes

The base line endocytosis activity of head kidney phagocytes was not different in cells from male or female fish but increased with size (R=0.20, $p<0.001$). Cells isolated from flounder in April showed a higher neutral red uptake compared to cells from fish collected in September ($p<0.001$). In addition, the variation of measurements was much higher in the spring samples. This was most obvious at Spiekeroog, but also at the other locations, HKL phagocytes from some individual flounder were highly active compared to the mean. At the Elbe and Outer Eider locations, the wide variation of the measurements was not as pronounced. Here the majority of cells showed a very low endocytosis activity, significantly less than at the other locations ($p<0.05$, Fig. 3). In autumn the variation of pinocytosis measurements were not as pronounced. Again, the activity of cells from Elbe flounder was decreased, but this could not be ascertained by

statistical tests. At the other locations, the activity varied within similar ranges and no site specific differences could be detected (Fig. 3).

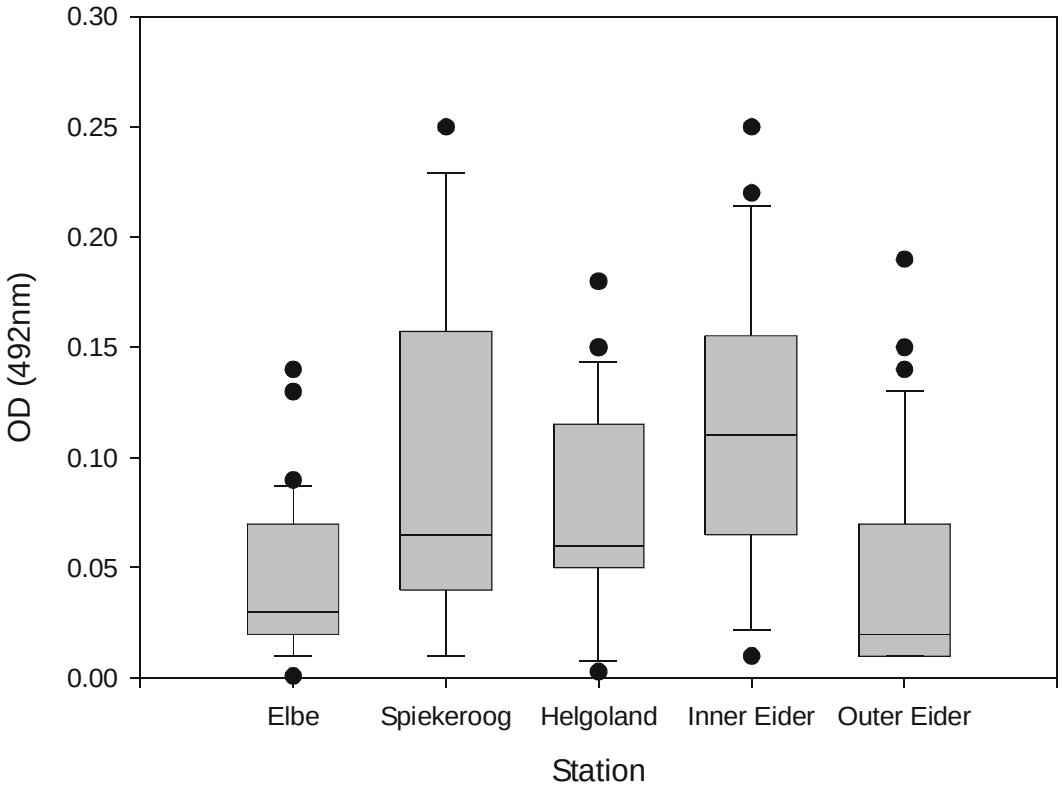


Figure 3: Endocytosis activity of head kidney derived leukocytes from flounder (*Platichthys flesus*) at 5 different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. n: see table 1. The activity of the cells was measured by means of neutral red uptake.
Cells from flounder collected at the Elbe location showed significantly lower endocytosis activity when compared to flounder from Spiekeroog, Inner Eider or Outer Eider ($p<0.05$). For key, see Fig. 1

The pinocytosis activity of HKL was positively correlated to DDT metabolites, the sum of PCBs and Dieldrin residues in the muscle of same individual (table 2). The pinocytosis activity of head kidney cells was positively correlated to other cellular responses of the innate immune system: HKL from flounder with increased pinocytosis activity responded with increased ROS production to PMA stimulation, elevated activity of macrophage aggregations in the liver, higher lysosomal stability and increased cholin-esterase activity in neurons. In addition, individuals with decreased pinocytosis activity of HKL significantly more often had increased ratios of DNA fragmentation (table 5).

Table 3: Cross-correlation between to parameters measured in flounder (*Platichthys flesus*) collected in 1999 and 2000 at 5 locations in the German Bight (*n*= 270-320, see table 1). Spearman's Correlation on Ranks: marked are correlations at p: <0.05:*, p<0.001: **

	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Haematocrit	1	0.11*	0.10	0.04	0.05
Lysozyme	0.11*	1	-0.07	0.12*	0.23**
Pinocytosis	0.10	-0.07	1	-0.10	0.16**
Basal ROS	0.04	0.12*	-0.10	1	0.58**
PMA activated ROS	0.05	0.23**	0.16**	0.58**	1

Table 4: Correlation between some heavy metal and chlorinated hydrocarbon residues in the muscle of flounder (*Platichthys flesus*) and applied parameters from the same individual. The flounder were in collected in 1999 and 2000 at 5 locations in the German Bight (*n*= 153-182, see table 1) Spearman's Correlation on Ranks: marked are correlations at p: <0.05:*, p<0.001: **. Residue data from K. Broeg and H. v. Westernhagen (Alfred Wegener Foundation, Bremerhaven)

Contaminant	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS	Ratio activated/ basal ROS
o,p-DDD	0.003	-0.18*	0.14	0.05	0.01	0.07
p,p-DDE	0.22**	-0.12	0.14	-0.07	-0.05	0.09
o,p-DDT	0.01	-0.09	0.16*	-0.06	0.13	0.07
p,p-DDT	0.006	-0.002	0.21**	-0.04	0.15*	0.17*
Dieldrin	0.002	-0.12	0.28**	0.15*	0.11	0.02
β-HCH	-0.04	-0.24**	-0.004	-0.06	-0.07	0.12
γ-HCH	0.08	0.17*	-0.12	0.08	-0.07	-0.06
PCB 101	0.15*	-0.16*	0.14	0.06	-0.02	-0,04
PCB 118	0.20**	-015*	0.21*	0.11	0.18*	-0.04
PCB 153	0.19*	0.11	0.24*	0.01	0.17*	0.17*
Sum PCB	0.22**	0.05	0.34**	0.01	0.20**	0.21**
Cu	0.09	0.06	-0.03	0.10	-0.20*	-0.19*

Table 5: Correlation between some physiological and biochemical parameters in flounder (*Platichthys flesus*) and the parameters applied here measured from the same individual. The flounder were collected in 1999 and 2000 at 5 locations in the German Bight (*n*= 242-322, see table 1) Spearman's Correlation on Ranks: marked are correlations at p: <0.05:*, p<0.001: **. The physiological and biochemical data are taken from Broeg (pers. communication) and Dizer *et al.* submitted. Abbreviations: *MAA*: macrophages aggregate area*; MAM*: macrophages aggregate activity; *Ly*: lysosomal stability; *EROD*: Ethoxyresorufine O-deethylase assay; *ChE*: cholinesterase activity; *DNA*: DNA unwinding assay; *Vit*: Vitellogenin

Biomarker	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
MAA	0.12*	0.04	-0.05	0.01	-0.05
MAM	-0.09	0.13*	0.17*	-0.06	0.23**
Ly	-0.04	0.15**	0.16**	-0.03	0.14*
EROD	0.16**	-0.21**	0.09	-0.16*	-0.14
ChE	-0.03	0.04	0.26**	-0.07	0.01
DNA	-0.22**	-0.07	-0.25**	0.17**	-0.05
Vit	-0.05	0.14*	-0.32**	0.24**	0.14*

Production of reactive oxygen species

The production of reactive oxygen species by head kidney leukocytes was not influenced by the sex of the specimen examined. It was, however, significantly influenced by the size of the fish: cells from larger fish had a higher base line as well as phorbol-ester-stimulated ROS production ($R = 0.14$, $p<0.01$). The base line respiratory burst was significantly ($p<0.001$) lower in spring compared to autumn samples (Fig. 4).

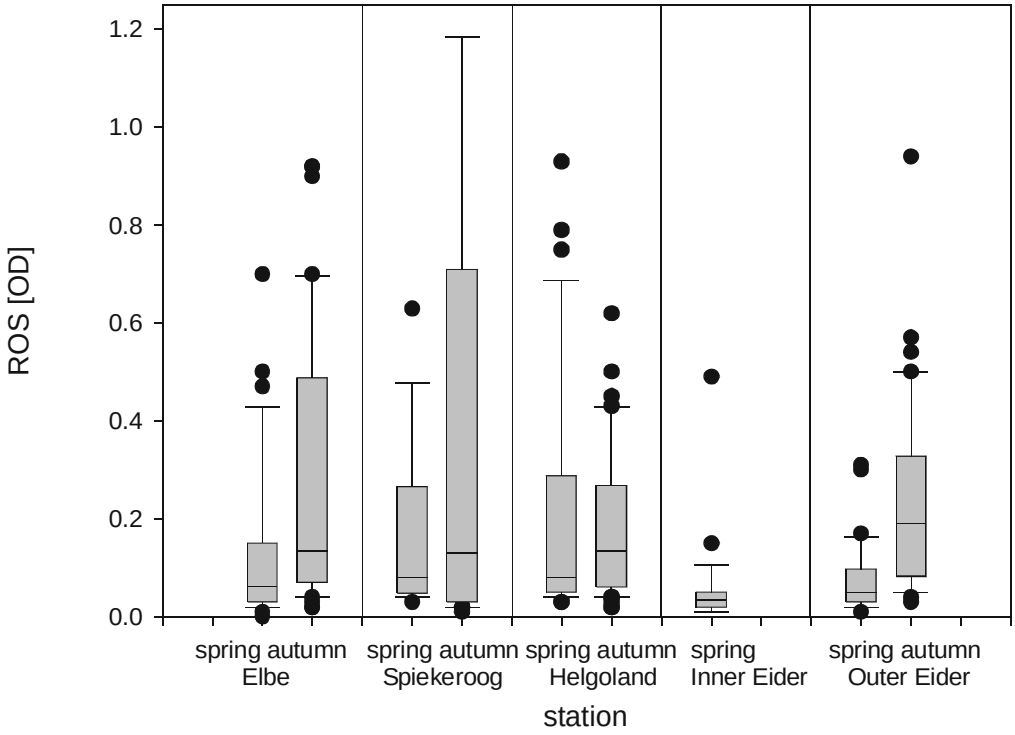


Figure 4: Basal production of reactive oxygen species by head kidney derived leukocytes collected from flounder (*Platichthys flesus*) at 5 different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. n: see table 1.
Differences in spontaneous ROS production between sites in spring and autumn (1999/2000).
There were no significant differences between the sites. For key, see Fig. 1

This difference was not observed in cells stimulated with the phorbol ester PMA (table 2). In spring, mean values of stimulated ROS production were similar at Spiekeroog, Helgoland and Outer Eider, and higher than the means at Elbe and Inner Eider. Head kidney phagocytes from some flounder could not be stimulated by PMA, while in other individuals, the cells responded with a high NBT reduction upon stimulation (Fig. 5). At Helgoland and Outer Eider, this resulted in a high variation of measurements in the spring sampling campaigns. In autumn, this variation was much lower. Then, cells from

flounder collected at the Elbe location produced significantly less ROS compared to cells from fish at Helgoland and Spiekeroog sites ($p<0.05$).

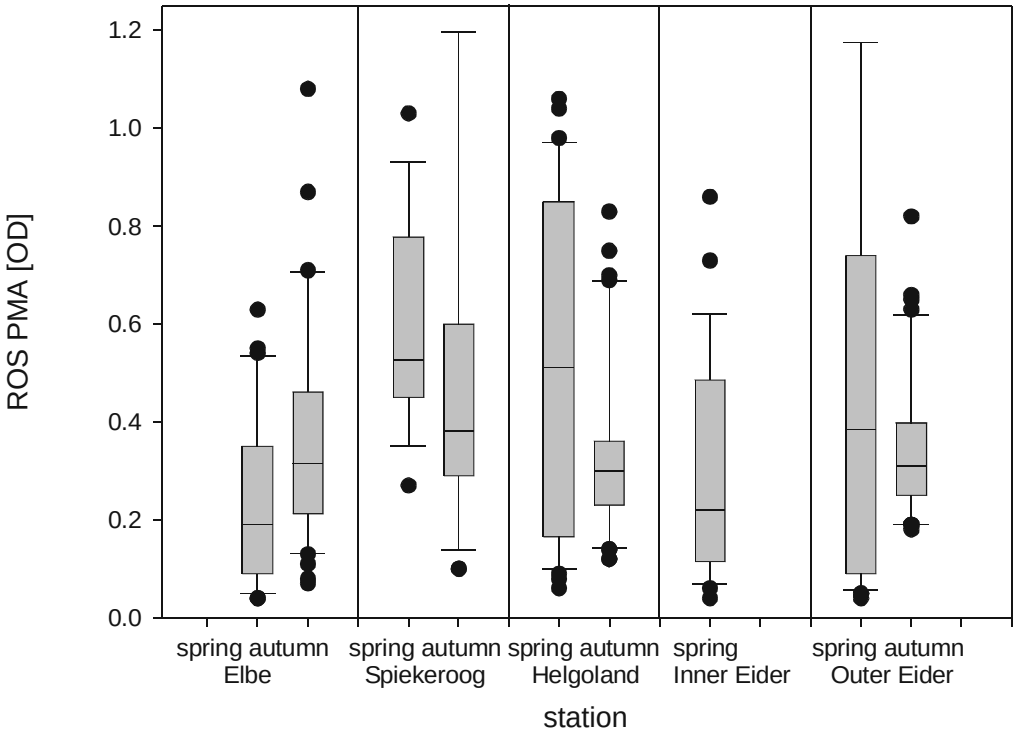


Figure 5: Production of reactive oxygen species by head kidney derived leukocytes upon stimulation with PMA collected from flounder (*Platichthys flesus*) at 5 different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. n: see table 1.
Autumn: sign. ($p<0.05$) lower values at Elbe compared with Helgoland and Spiekeroog. sign. ($p<0.05$) lower values at Inner Eider than Spiekeroog.
Spring: no sign. differences between sites. For key, see Fig. 1

When considering the ratio of ROS production upon stimulation versus base line ROS production, cells from flounder collected at both of the Eider locations had significantly higher ratios compared to Elbe flounder (Fig. 6).

The respiratory burst activity of HKL was correlated to residues of several chlorinated hydrocarbons and to heavy metals in the muscle of the same individual examined. The base line ROS production was positively correlated with Dieldrin residues. The ROS response to phorbol ester stimulation was positively correlated to DDT and PCB residues in the muscle of the same individual, and there was a negative correlation to copper residues. The ratio of stimulated ROS versus base line ROS production showed a positive correlation to DDT, PCB and Hg residues, while this ratio was reduced along with increasing Cu residues. The PMA activated ROS production of HKL was

correlated to other indicators of innate immune response, such as activity of macrophage aggregates and lysosomal stability in the liver. Interestingly, base line ROS production was lower in individuals with increased induction of the EROD system in liver cell, while this was not seen in PMA-activated cells (table 5).

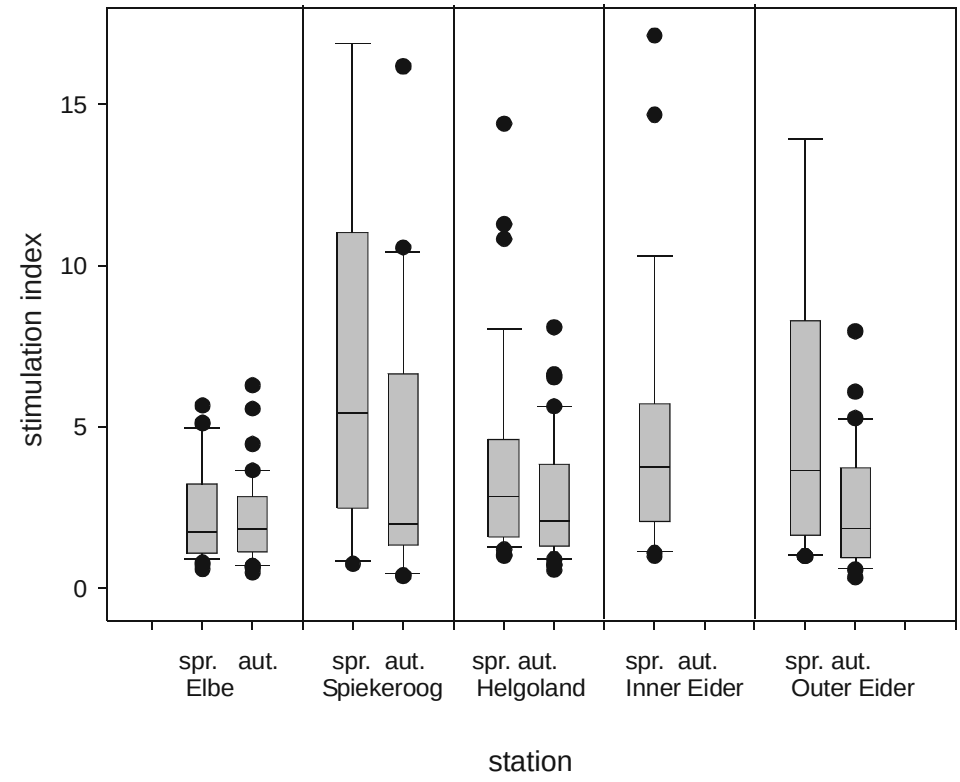


Figure 6: Production of reactive oxygen species by head kidney derived leukocytes from flounder (*Platichthys flesus*) at 5 different sampling sites in spring and autumn sampling campaigns in 1999 and 2000. Ratio of ROS production upon PMA stimulation / basal production. n: see table 1.
Cells from flounder collected at Spiekeroog, Outer Eider and Inner Eider in spring had significantly higher values ($p<0.05$) than cells from Elbe flounder. In autumn no significant differences were observed. *spr.*: spring; *aut.*: autumn. For key, see Fig. 1

Discussion

From laboratory studies, it has become clear, that environmental contaminants indeed modulate immune responses in fish (for reviews see Dunier and Siwicki, 1993, Zelikoff 1993, Bols *et al.* 2001). However, with attempts to extrapolate experimental data to "field" situations in monitoring studies, problems arise. In natural environments, fishes are exposed to an undefined cocktail of various substances for an unknown period of time. To gain knowledge about the actual contamination level of the individuals studied, the present study was substantiated by extensive analytical chemistry of sediment as

well as fish residues, which included chlorinated hydrocarbons and heavy metals (Broeg in preparation, Dizer *et al.* submitted). In a previous study (Broeg *et al.* 1999) on flounder from the same locations, a contamination gradient with respect to residues of chlorinated hydrocarbons was observed with highest values in fish from the Elbe estuary and lowest values in fish from the Tiefe Rinne near Helgoland. This gradient could be established when the residue data were based on the fat content of the liver, which is considered to reflect the chemical burden of the habitat (Broeg *et al.* 1999). From sediment analysis, however, such a clear gradient could not be established and when residue data from flounder based on wet weight were considered for analysis, individual flounder could not be related to different sample sites by means of residual analysis (Schmolke *et al.* 1999). This most probably is a consequence of a strong reduction of heavy metal as well as PCB influx into the southern North Sea during the last decade, which resulted in a decrease of heavy metal and PCB content in the sediment between 45 and 85 % (De Jong *et al.* 1999). In the German Bight of the southern North Sea residues of these substances now are far below previous levels and far below contamination levels of other marine sites such as the Mediterranean coast near Haifa in Israel (Kress *et al.* 1999).

When considering heavy metal and chlorinated hydrocarbon contaminations in flounder muscle, very low residues were found in animals from all the sites, and there was no "clean" site with all the residues "below detection limit" (Schmolke *et al.* 1999, Broeg pers. communication). In this situation, when analysing immunological data in their response to pollution, clear differences between sampling locations which could be confirmed in all the sampling campaigns could not be established in the present study. This mainly was an effect of the high variation in plasma lysozyme level as well as for phagocyte activity at some of the locations. The variation of measurements most probably was a result of different contamination profiles of individual flounder: some correlations were found between chlorinated hydrocarbon levels of individual flounder and innate immune responses measured from the same individuals. It also has to be taken into account that contaminants may cause indirect effects such as elevated levels of cortisol, which have a marked modulatory potential of immune functions (Bennet and Wolke 1987 a, b). Stimulatory effects of contaminants, as observed in the present study, may also be a consequence of indirect effects of contaminants (Faisal *et al.* 1991). Some contaminants, however, such as copper in the present study, are clearly immunotoxic.

Cellular immune responses are considered as sensitive indicators of biological effects of pollutants (Secombes *et al.* 1997). Broeg *et al.* (1999) showed that the stability of hepatocyte lysosomes was modulated in a delicate manner. Thus the integrity of hepatocyte lysosomes provided valuable information for the interpretation of the expression of cytochrome P450 1A in liver cells of the same individual. Likewise, we found a correlation between lysosomal stability of liver cells and the activity of plasma lysozyme as well as head kidney phagocytes of the same individuals, indicating an overall impairment of cell functions with decreased lysosome stability.

Experimental studies showed that flounder acquire contaminants with the food rather than by passive uptake via skin or gills (Mondon *et al.* 2001). Thus contaminant residues in benthic invertebrates such as *Mytilus edulis,* which serve as prey for flounder, most probably have a higher biological relevance than residue measurements from the sediment. This is supported by the finding, that flounder from a location with less contaminated *Mytilus* had a higher plasma lysozyme activity.

In conclusion, the results presented here underline that biological effects of environmental contaminants can be monitored by means of immunological assays in the "field". In a complex environment such as the German Bight of the North Sea, with a diffuse contamination of various compounds at a low level, it was however difficult to clearly separate polluted from less affected sites, most likely because contaminated individuals were found at all locations. When the contamination load of individual flounder were considered, it was possible to spot pollution mediated effects. The present study was part of an integrated monitoring program on flounder, which showed that in conjunction with other physiological data from the same individual, innate immune parameter also allowed to observe pollution effects. Cellular function such as uptake of neutral red was impaired in individuals with increased proportions of DNA adducts or decreased stability of lysosomes. The activity of plasma lysozyme also was decreased in individuals with impaired lysosome stability, and showed some correlation to cytochrome P 450 1A induction. This underlines that innate immune parameters such as plasma lysozyme activity or phagocyte functions form valuable parameters as parts of an integrated monitoring program.

Chapter 5

The effect of parasite infection on the innate immune response of European flounder (*Platichthys flesus* L.) in the southern North Sea

Summary

The infection of European flounder (*Platichthys flesus* L.) with different parasites did not result in alteration of innate immune response. Due to high variability in infection state and also high variability in immune function no dependencies were obvious. The data pointed out that the most abundant parasites show no influence on immune responses measured here, which indicates, that these parameters are not sensitive to parasite infection. The immune parameters considered here are regarded as promising indicators of chemical contaminant induced variation of piscine immune responses, which could be implemented in pollution monitoring programs.

Introduction

World-wide the aquatic environment is abused by the introduction of a high number of xenobiotic compounds derived from human activities in industry and agriculture. Many of these substances have the potential to impact on the ecosystem at relatively low concentrations (Conell *et al.* 1999). In order to assess the risk of contaminant exposure for organisms and to classify the environmental health of an ecosystem under challenge, innate immune responses in fish are recommended as bioindicators of xenobiotic exposure (Bols *et al.* 2001). In laboratory or mesocosm studies, the immunomodulatory potential of a wide variety of compounds, such as hydrocarbons or heavy metals was assessed (for review see Dunier and Siwicki 1993, Zelikoff *et al.* 2000). In these experiments hatchery or laboratory raised fish of known health status were exposed to specific compounds at defined concentrations for a limited period of time (e.g. Zelikoff *et al.* 2000, for review see Bols *et al.* 2001). In 'real world' monitoring programmes, fishes were collected at coastal or marine sites for an assessment of effects of a largely unknown mixture of contaminants (Secombes *et al.* 1997, Zelikoff *et al.* 1997). In many cases, biomarker responses varied within wide ranges, which made interpretation of biological effects difficult. Besides genetic heterogenicity, some variation might originate from differences in life history and parasite infection in individuals examined. In coastal or marine environments, most fish individuals harbour a very divers parasite fauna (Overstreet 1997 , Schmidt *et al.* submitted, Broeg *et al.* 1999), and infections with some species might result in a modulation of physiological or immune responses (Overstreet 1997), which are considered as indicators of biological effects of environmental contaminants. In most studies carried out in the past physiological or innate immune response were only evaluated in regard to chemical residues found in the

fish or at the sample site (Tahir *et al.* 1993, Secombes *et al.* 1995, 1997) and nonpathogenic infections with parasites for instance were not considered. In an integrated biological effect monitoring program on flounder, *Platichthys flesus*, in addition to some innate immune responses, the parasite fauna of the same individuals was assessed (Chapter 4, Schmidt *et al.* submitted). This allowed a correlation of immunological measurements to the parasitological findings.

Material and methods

Sampling

In April and September 1999 and 2000 a total of 331 flounder (*Platichthys flesus* L.) were collected at five different locations in the German Bight (Elbe estuary, Inner Eider estuary, Outer Eider, Spiekeroog and Tiefe Rinne near Helgoland), during cruises with the research vessel "Uthörn" of the Alfred Wegener Institute. Details of sampling, examination of the flounder (Chapter 4) and collection of parasites were described elsewhere (Schmidt *et al.* submitted). For analysis only macroscopically healthy flounder of the size class 18-25 cm were used.

Examination procedure

On board of the research vessel, body length and weight from each were fish were measured, and macroscopic visible ectoparasites were collected. Blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-heparin bead (Sarstedt, Germany). From the blood, the haematokrit was determined according to standard procedures (Houston 1990). The remaining blood then was transferred to centrifugation tubes, centrifuged at 2000 x g for 15 min at 4 °C, and the supernatant plasma was collected and frozen at –80 °C. Then the fish was killed, dissected and the head kidney was removed and transferred into a centrifugation tube filled with wash medium (RPMI medium supplemented with 10 000 IU l^{-1} sodium heparin, medium: Biochrom, Berlin, Germany, heparin: Sigma-Aldrich, Germany) and stored at 4° C for up to 24 h for further processing. In addition liver, kidney, intestine, gills and muscle samples were collected for parasitological, physiological and biochemical research as well as residual analysis. The methods and results of these are reported elsewhere (Broeg in preparation, Schmidt *et al.* submitted, Dizer *et al.* submitted). From the morphological measurements, a whole body condition factor (CF) was determined for each fish according to the formula:

$$CF = (\text{body weight in g} / \text{body length in cm}^3) \times 100$$

and used as an allometric index for overall health (Busacker *et al.* 1990).

Parasitological examination

On board, the flounder were examined for ectoparasites. Specimen were collected from the skin and stored in 70 % ethanol for further counting and identification. Fresh smears were taken from skin, gills and nose cavity, gut and gall bladder epithelium and immediately examined for the presence of parasites by light microscopy. Then the fish were killed and dissected. Gills then were fixed in 4 % buffered (pH 7.2) formaldehyde solution. The gut was removed, opened and transferred to saline solution (0.9 % NaCl) and a drop of detergent was added. Under these conditions, parasites detached from the intestinal tissue and settled at the bottom of the vial. Then the supernatant fraction was discarded, the sediment resuspended in saline and again allowed to settle for a few minutes. After three washes organic waste was removed from the gut contents. The remaining parasites were fixed in 70 % ethanol for further investigation. Then gut, kidney, gall bladder and gills were fixed in 4 % buffered formaldehyde solution. Transverse sections of mid- and hind-gut as well as small parts of kidney were taken for histological investigation. Gills, gut and gut contents were examined for metazoan parasites with a dissection microscope. Parasites were collected, counted and stored separately for individual fish. Sections of gut and kidney were processed by standard histological procedures (Romeis 1989), stained by Giemsa's technique and examined with a light microscope for tissue invading parasites.

For identification of macroparasites, individuals were cleared in 80-90 % lactic acid, mounted in glycerine-jelly and observed with the microscope. Smears of *Trichodina* spp. were air dried and stained by Klein's silver impregnation method (Lom and Dyková 1992).

The identification of parasites was done using standard literature (Yamaguti 1959, 1963, 1971, Kabata 1979) and with support by Dr. M. Køie (Marine Laboratory, Helsingør, Denmark) for trematodes, cestodes and acanthocephalans and by Dr. F. Moravec (Institute of Parasitology, Ceské Budejovice, Czechian Republic) for nematodes.

All parasitological examinations were conducted by the working group of Prof. Dr. W. Körting (Tierärztliche Hochschule Hannover) and data were kindly shared for this analysis (for details: Schmidt *et al.* submitted).

Lysozyme assay

Lysozyme activity of flounder plasma was determined by means of a turbidimetric assay according to Parry *et al.* (1965). A suspension of 0.2 g l^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl of flounder plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and after 4.5 min at a temperature of 20±2 °C. The decrease of absorbance was used to calculate lysozyme activity. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 min^{-1}. Hen white egg lysozyme (Sigma-Aldrich, Germany) was used as external standard as described by Hutchinson and Manning (1996).

Leukocyte isolation

Leucocyte isolation was done as described previously (chapter 2). Briefly, cell suspensions of head kidney leucocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zurich, Switzerland). Isolated HKL were washed three times with wash medium at 550 x g for 10 min and resuspended in cell culture medium (RPMI-1640 supplemented with 100 000 IU l^{-1} penicillin, 100 mg l^{-1} streptomycin and 4 mM L-glutamine and 1% [v/v] carp serum (chemicals: Biochrom, Berlin, Germany; carp serum: serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -20 °C until use). Numbers of viable cells were determined by trypan blue exclusion in a Neubauer haemocytometer.

Production of reactive oxygen species by head kidney leukocytes

Generation of reactive oxygen species (ROS) by head kidney leukocytes (HKL) was measured by means of the nitro blue tetrazolium salt (NBT) reduction assay after cell isolation, as described earlier (Chapter 2). Briefly, cells were incubated in 96-well flat-bottom microtiter plates (10^6 cells in a final volume of 175 µl of cell culture medium) in triplicate and their ROS production was induced by adding 0.15 mg l^{-1} phorbolmyristate acetate (PMA). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the spontaneous ROS generation of cells. After incubation for 2 h at 18 °C, the supernatants were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol.

Methanol was removed and the fixed cells were air dried over night and stored in the dark for up to two weeks. The reduced NBT (formazan) was dissolved in 125 µl of 2 M KOH and 150 µl DMSO per well (All chemicals: Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm.

Endocytosis activity of head kidney phagocytes

Endocytosis activity of head kidney phagocytes was measured by means of neutral red retention of isolated head kidney cells as described by (Mathews *et al.* 1990). This assay was adapted to microtiter plates. Briefly, 10^6 cells were incubated in a final volume of 175 µl culture medium for 2.5 h at 18 °C with 10 mg l^{-1} neutral red (NR, Sigma-Aldrich, Germany). All set-ups were made at least in triplicate. After incubation each well was washed two times with 125 µl phosphate buffered saline (PBS). After removing the PBS, the cells were air dried over night and frozen at -20 °C for up to two weeks. For spectrophotometric readings the cells were lysed with 100 µl acid ethanol (3 % HCl in 95 % Ethanol) and mixed with 100 µl PBS. The optical densities were recorded at 492 nm.

Statistics

Normality of the data was tested with the Kolmogorov-Smirnow test. To determine the significance between groups, data were compared by Student's t-test, Mann-Whitney's rank sum test, or by Kruskal-Wallis ANOVA and subsequent multiple comparison by means of the Student-Newman-Keuls method at a probability of error $p<0.05$. Correlations between data sets were tested by Pearson's product moment correlation test or by Spearman's rank correlation test. Correlations were considered as significant at a probability of error $p<0.05$. The analyses were carried out using SigmaStat® 2.0 and STATISTICA 6 (StatSoft) software packages.

Results and conclusion

A total of 17 parasite species/taxa were present at all sampling sites, but not all of them were found during both seasons or during each sampling campaign. A list of the parasites, their prevalence and abundance is shown in table 3, for details see Schmidt *et al.* (submitted).

Table 1: Summary of the sampling program on flounder (*Platichthys flesus*) caught for analysis of innate immune parameter and parasites in 1999 to 2000. All parameter were measured from same individual.

Site / campaign	Spring 1999	Autumn 1999	Spring 2000	Autumn 2000
Elbe	20	20	20	20
Spiekeroog	9	20	9	20
Helgoland	20	20	20	20
Inner Eider	15	-	18	-
Outer Eider	19	20	20	20

Only 6 species/taxa were regularly present and sufficiently abundant over the whole sampling period: the Ciliophora *Trichodina* spp., the copepods *Acanthochondria cornuta*, *Lepeophtheirus pectoralis* and *Lernaeocera branchialis*, the helminths *Zoogonoides viviparus* and *Cucullanus heterochrous*. Thus the analysis in respect to a modulation of innate immune parameters was focussed on these species. Species of Microsporea, Myxozoa and Apicomplexa, which previously were reported to influence immune responses in fish (Munoz *et al*. 2000, Leiro *et al.* 2001, Steinhagen *et al*. 1998), were present in low infection intensities only but they also were considered for analysis.

The examination of the parasite load of flounder revealed some relations among the parasite groups. Individuals with high numbers of helminth were significantly less infected with *Trichodina* sp. and Myxozoa (tab. 2). Infections with *Epieimeria* sp. (Apicomplexa) were concomitant with *Trichodina* infections and individuals infected with Myxozoa often were found associated with Microsporea and Trichodina ($p<0.05$, $p<0.01$ respectively, tab. 2).

The presence or absence of parasites had a marked impact on several of the innate immune parameter measured here, but the pattern varied with the single parasite species (table 4). Individuals with *Trichodina* sp. infection had reduced plasma lysozyme levels with 990 (767-1348) IU ml^{-1} compared to 1172 (892-1519) IU ml^{-1} in non infected fish. In flounder with a renal myxosporidian infection, the basal ROS production was decreased with 0.039 (0.025-0.143) compared to 0.093 (0.044-0.262) OD values in not affected individuals. In individuals with high abundance of copepods (more than 20 copepod individuals per fish, n=246) the plasma lysozyme activity and the basal as well as PMA triggered ROS production of HKL were significantly increased. Individuals with high copepod load had a plasma lysozyme activity of 1128 (856- 1461) IU ml^{-1}, a basal ROS production of 0.0965 (0.045- 0.261) OD and a PMA stimulated ROS production of 0.342 (0.215-0.590) OD values. In individuals with low copepod load (<20 copepod individuals, n=56) the lysozyme activity was 875 (602- 1234) IU ml^{-1},

and the respiratory burst activity was 0.057 (0.029- 0.181) OD and 0.216 (0.103- 0.368) OD, respectively.

In individuals with microsporidian infections or with *Myxidium incurvatum*, the innate immune responses monitored here appeared not to be affected when compared to measurement from individuals not infected by these parasites.

Table 2: Cross-correlation between parasites from flounder (*Platichthys flesus*) collected in 1999 and 2000 at 5 locations in the German Bight (*n*= 270-320, see table 1) Spearman's Correlation on Ranks: marked are correlations at p: <0.05:*, p<0.01: **; Apicomplex: *Epieimeria* sp.; Microporea: *Glugea stephani*; Ciliophora:*Trichodina* spp.; Myxozoa: *Myxidium incurvatum*; Sum copepoda: Sum of *Acanthochondria cornuta*, *Caligus elongus*, *Holobomolochus confusus*, *Lerneocera branchialis*, *Lepeophtheirus pectoralis*; Sum helminth:gut dwelling parasites of monogenea and digenean trematodes, cestodes, nematodes and acanthocephalan. Data from Schmidt *et al.* (submitted)

	Apicomplea	Microporea	Ciliophora	Myxozoa	Sum copepoda	Sum helminth
Apicomplea	1					
Microporea	-0.01	1				
Ciliophora	0.19**	<0.01	1			
Myxozoa	0.01	0.11*	0.16**	1		
Sum copepoda	0.02	-0.06	-0.09	0.06	1	
Sum helminth	0.02	-0.03	-0.27**	-0.13*	0.12	1

Table 3: List of parasites species, target organ/tissue, prevalence and infection intensity recovered from flounder (*Platichthys flesus*) in the German Bight during sampling campaigns in spring and autumn from 1999-2000 (data from Schmidt *et al.* submitted).

Taxonomic group	Parasite species	Target organ/ tissue	prevalence	intensity
Apicomplexa	*Epieimeria* sp.	gut	24.3±20.1	
Ciliophora	*Trichodina* spp.	gills	54.2±31.8	
Microsporea	Microsporea sp.1	kidney	30.3±10.3	
	Glugea stephani	gut	0.7±2.0	
Myxozoa	Myxozoa sp. 1	kidney	9.5±11.0	
	Myxidium incurvatum	gall bladder	23.6±17.5	
Digenea	*Derogenes varicus*	gut	6.0±9.0	1.5±0.9
	Brachyphallus crenatus	gut	4.5±6.6	5.1±9.5
	Zoogonoides viviparus	gut	17.1±27.2	56.1±68.6
	Lecithaster gibbosus	gut	1.5±3.2	
	Podocotyle atomon	gut	5.6±7.5	3.5±5.5
	Metacercaria sp. 1	gills	60.7±22.6	52.5±132.8
Cestoda	*Bothriocephalus* spp.	gut	0.8±1.9	1.0±0
	Proteocephalus sp.	gut	0.8±2.6	4.3±4
	Cestoda larvae sp. 2	gut	0.3±1.2	5.0±0.0
Nematoda	*Paracapillaria gibsoni*	gut	16.6±17.1	14.2±30.7
	Cucullanus heterochrous	gut	46.2±20.8	3.1±4.2
	Dichelyne minutus	gut	5.8±8.3	1.2±0.4
	Goezia sp.	gut	1.4±3.8	1.8±0.8
	Hysterothylacium aduncum	gut, liver	12.9±12.0	2.9±5.5
Acanthocephala	*Corynosoma sp.*	gut	3.7±4.2	1.4±1.4
	Echinorhynchus gadi	gut	3.8±12.0	1.3±0.5
	Pomphorhynchus laevis	gut	0.4±1.6	23±0.0
Copepoda	*Acanthochondria cornuta*	gill cavity	67.8±28.1	7.8±6.4
	Caligus elongatus	skin	4.8±10.2	2.6±3.2
	Holobomolochus confusus	nose cavity	3.9±7.4	1.1±0.5
	Lernaeocera branchialis	gills	95.0±15.4	61.7±55.6
	Lepeophtheirus pectoralis	skin, fins	76.3±27.7	10.4±7.3

Table 4: Comparison of the presence parasites in flounder (*Platichthys flesus*) and immunological parameters measured from the same individual. The flounder were collected in 1999 – 2000 at 5 different locations in the German Bight, North Sea. Compared were immune parameters of affected flounder to measurements obtained from not infected individuals by means of the Mann-Whitney rank sum test. Listed are p values obtained from the test. For sum copepoda and sum helminth correlation were conducted. Shown is the Spearman rank correlation coefficient. Statistically significant differences in the immune response between the groups at $p<0.05$ are marked in **bold**. (+): increased immune parameter in infected flounder; (-): depressed immune parameter in infected individuals. n=286-330; Myxozoa: Myxozoa sp. (kidney); Sum copepoda: Sum of *Acanthochondria cornuta*, *Caligus elongus*, *Holobomolochus confusus*, *Lerneocera branchialis*, *Lepeophtheirus pectoralis*; Sum helminth: sum of all gut dwelling parasites of monogenea, digenea, cestoda, nematoda and acanthocephalan. Parasite data from Schmidt *et al.* (submitted)

Parasite	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Microsporea sp.	0.75	0.06	0.34	0.83	0.89
Trichodina spp.	0.95	**0.02(-)**	0.12	0.17	0.14
Myxdium incurvatum	0.41	0.07	0.43	0.14	0.56
Myxozoa	0.26	0.14	0.24	**<0.01(-)**	0.25
Sum copepoda	0.01	**0.12**	0.11	0.02	0.10
Sum helminth	0.09	**0.19**	0.02	0.11	0.10

Table 5: Multiple linear regressions between parasites in flounder (*Platichthys flesus*) and the immune parameter measured in the same individual. The presence of parasites is tested as explaining variable on the immune responses applied here. Given are the p values obtained from the calculation. An influence was considered to be significant at $p<0.05$*; $p<0.001$**. n=286-330. Apicomplexa: *Epieimeria* sp.; Microporea: *Glugea stephani*; Ciliophora:*Trichodina* spp.; Myxozoa: Myxozoa sp.; Sum copepoda:Sum of *Acanthochondria cornuta*, *Caligus elongus*, *Holobomolochus confusus*, *Lerneocera branchialis*, *Lepeophtheirus pectoralis*; Sum helminth:gut dwelling parasites of monogenea, digenea, cestoda, nematoda and acanthocephalan. Parasite data from Schmidt *et al.* (submitted)

Parasite	Haematocrit	Lysozyme	Pinocytosis	ROS	PMA activated ROS
Apicomplexa	0.67	0.85	<0.01*	<0.01*	0.09
Microporea	0.21	0.14	0.21	0.44	0.93
Ciliophora	0.75	0.30	0.12	0.81	0.37
Myxozoa	0.34	0.34	0.19	0.23	0.12
Sum copepoda	0.89	0.14	0.02*	0.60	0.52
Sum helminth	<0.01*	<0.01**	0.35	0.77	0.12

High infections with helminths, calculated as sum of all gut dwelling helminth parasites such as monogeneans, digeneans, cestodes, nematodes and acanthocephalan, had an impact on the innate immune response measured here ($r=0.19$, $p<0.05$), but when the data plots for this relation were analysed, a clear correlation could not be visualized. The majority of the flounder individuals harboured low sums of helminths only. In these individuals, the measurements of the immunological parameters already varied over the whole range. This picture could be generalized for other parasites in this study as well. The majority of the flounder individuals carried low infections, and in these individuals, the measurements of immune parameters varied in the same ranges as observed for flounder with high parasite loads. These findings indicate that in flounder individuals examined here a sub-clinical parasite infection most likely had a little impact only on the assessed immune parameters. This assumption is also supported by a multiple regression analysis. When the abundance of parasites was considered as influencing factors on the immune parameters applied here, haematocrit ($p<0.01$) and the plasma lysozyme activity ($p<0.001$) appeared to be mainly affected by the presence of gut dwelling helminths while head kidney phagocytes (pinocytosis and basal respiratory burst activity, $p<0.01$) were found to be mainly influenced by the presence of an *Epieimeria*-infection (table 5). It is surprising that parasites with low abundance such as *Epieimeria* or the sum of helminths in a multiple regression analysis appear as a significant explaining factor, while copepods as the most abundant parasite group does not seem to play a role as an influencing factor on the innate immune responses measured here.

Taken together, the data presented here suggest that the sub-clinical parasite load of flounder caught from a marine site has no major modulatory impact on innate immune functions of the individual. Thus, when these immune parameters are considered as biomarkers for the immunomodulatory potential of environmental contaminants, sub-clinical parasite infections most probably will not modulate the measurements. We are aware, that this only is true for those parasites found here and not for other pathogens such as bacteria, viruses or fungi. In the present investigation, most pathogenic species were excluded by selecting externally healthy individuals only. In conclusion, for an assessment of the immunomodulatory potential of environmental contaminants, clinically healthy fish show little alteration of innate immune responses by parasite infections.

Chapter 6

Assessment of some innate immune response in dab (*Limanda limanda* L.) from the North Sea and the Baltic Sea as part of an integrated biological effect monitoring

Summary

The marine flatfish dab (*Limanda limanda* L.) that comes into direct contact with contaminated sediments is frequently used as sentinel species in international monitoring programmes of biological effects of contaminants in coastal waters. As indicators of sublethal chronic effects of contaminants, in this study immune responses were recorded in addition to measurement of the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) in liver cells, the inhibition of acetylcholin esterase (ACHE) in muscle and a recording of grossly visible diseases and parasites. In total 336 dab were analysed from 5 sampling areas in the North Sea, which included a location in the German Bight, the Dogger Bank, a location in the Firth of Forth, Scotland and 2 locations close to oil and gas platforms. When considering plasma lysozyme levels, pinocytosis and respiratory burst activity of head kidney leukocytes, a clear gradient could be observed with decreased measurements in individuals collected from the Firth of Forth and locations near the oil or gas platforms compared to dab from the Dogger Bank or the German Bight. Individuals with induced EROD activity displayed reduced lysozyme and respiratory burst activities. In dab infected with the lymphocystis virus or with nematodes, lysozyme levels also were reduced. The data obtained here indicate that the assessment of innate immune parameters in a monitoring programme provides supplementary information about immunomodulatory effects associated with exposure of fish to contaminants. Especially plasma lysozyme, which can be analysed in an easy and inexpensive assay, will be a good parameter in a battery of other bioindicators.

Introduction

World-wide the aquatic environment is abused by the introduction of a high number of xenobiotic compounds derived from human activities in industry and agriculture. Many of these substances have the potential to impact on ecosystem on relatively low concentrations (Conell *et al.* 1999). In order to assess risk of contaminant exposure for organisms and to classify the environmental health of an ecosystem under challenge, various monitoring techniques were used (Van der Oost *et al.* 1997). These biomarkers are biochemical, physiological, or histopathological indicators of exposure to anthropogenic substances which occur at exposure to concentrations less than those causing adverse toxicological effects. In various animals, the immune system appears to be exquisitely sensible to toxic effects of chemicals of environmental concern. In the

mammalian system, a battery of well-characterised immune assays to test for functional or histopathological parameters are available (Luster and Rosenthal 1993) and for fish, many of the same endpoints were used in laboratory studies to demonstrate chemically-induced immunotoxicity in laboratory fish (recent reviews by Zelikoff *et al.* 2000, Bols *et al.* 2001).

In field studies on "real-world" polluted aquatic environments, the international committee for the exploration of the sea (ICES) recommended monitoring programmes of biological effects of contaminants on coastal environments by means of biochemical parameters such as the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) in liver cells or the inhibition of acetylcholin esterase (ACHE) in muscle in addition to grossly visible fish diseases and parasites (ICES 1996, 1999). As sentinel species, marine flatfishes are frequently used in international monitoring programmes. In the North Sea and the Baltic, this is dab (*Limanda limanda* L.) and European flounder (*Platichthys flesus* L.) mainly (Grinwis *et al.* 2000, Lang and Mellergard 1999, Lang *et al.* 1999, Secombes *et al.* 1997, Broeg *et al.* 1999). For immune function assessment, studies reveal that contaminants modulate immune parameters in fish (Arkoosh *et al.* 1994, Secombes *et al.* 1995), but integrated studies, which correlate immune functions to measurements of biochemical biomarkers, for instance are scarce.

In the present study, innate immune functions were assessed from dab collected at various locations in the North Sea along a pollution gradient. From the same individuals, biochemical biomarkers of pollution and grossly visible diseases were recorded according to ICES recommendations (ICES 1996, 1999) and the findings of the different measurements were compared.

Material and methods

Sampling

Sampling was carried out on board of the research vessel "Walther Herwig III" from the German Federal Research Centre for Fisheries during the 209th, 220th and 231st cruises, conducted in August and September 1999, 2000 and 2001. Fishing was carried out using standardised fishing methods (for details see Lang and Mellergaard 1999) at 6 different locations in the North Sea and the Baltic Sea. The location of the sampling sites and their geographical positions are shown in Fig. 1. In the North Sea, regions at P01 and P02 are characterised by oil and gas platforms, station N06 is located in the Firth of Forth, N04 at the Dogger Bank and JMP near Helgoland in the German Bight.

In the Baltic Sea, dab were sampled at B01, in the Kiel Bight during the 1999 and 2000 cruises.

On board of the research vessel, fish were sorted out immediately and kept in tanks with permanent seawater flow-through and aeration. Further processing took place within one hour. In total, 336 female dab (*Limanda limanda* L.) of the size class 20-24 cm were used for this investigation. Per site and campaign a maximum of twenty fish were collected and processed.

Sediment samples were taken during in 1999 during the 209th cruise at the six different sites and analysed for organic contaminants. Methods and results of this analysis are described in detail by Kammann *et al.* (2001).

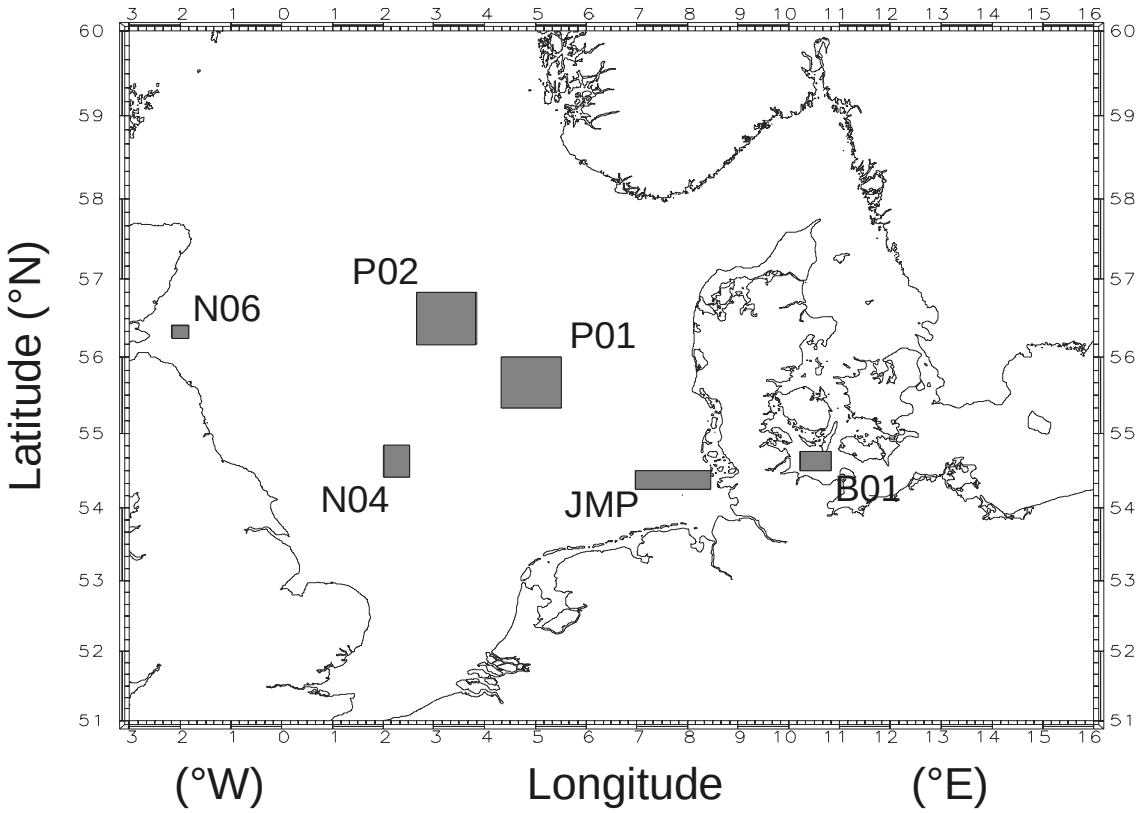


Figure 1: Locations of sampling sites for dab (*Limanda limanda*) in the North Sea and Baltic Sea. P01 (55°20’ N- 56°00’ N, 04°20’E- 05°30’ E) and P02 (56°10’ N- 56°50’ N, 02°40’E- 03°50’ E): Oil and gas platforms (“Ekofisk complex” and “Dan Oil field”); N04 (54°25’ N- 54°50’ N, 02°00’ E- 02°31’ E): Dogger Bank; N06 (56°15’ N- 56°25’ N, 01°50’ W- 02°10’W): Firth of Forth; JMP (54°15’N-54°30’N, 06°58’ E- 08°27’ E): German Bight; B01(54°30’ N- 54°45’ N, 10°13’ E- 10°50’ E): Kiel Bight.

Examination procedure

On board of the research vessel, body length and weight were measured from each fish. Blood was drawn from the caudal vein into disposable syringes prefilled with a lithium-

heparin bead (Sarstedt, Germany). From the blood, the haematocrit was determined according to standard procedures (Houston, 1990). The remaining blood then was transferred to centrifugation tubes, centrifuged at 2000 x g for 15 min at 4 °C, and the supernatant plasma was collected and frozen at –20 °C. Then the fish were inspected for the presence of externally visible diseases and parasites using a standardised method as described by Lang *et al.* (1999). The presence or absence of following diseases and parasites was registered as described by Dethlefsen *et al.* (2000): lymphocystis, epithelial papilloma, acute or healing skin ulcer, pigmentary abnormality, infection with nematodes and acanthocephalans. The otoliths were prepared for age determination (see also Drevs *et al.* 1999).

Then the fish were killed, dissected and the head kidney was removed and transferred into a centrifugation tube filled with wash medium (RPMI medium supplemented with 10 000 IU l^{-1} sodium heparin, medium: Biochrom, Berlin, Germany, heparin: Sigma-Aldrich, Germany) and stored at 4 °C for up to 24 h for further processing.

In addition liver and muscle samples were collected from the same individuals for biochemical analysis.

Biochemical parameters (EROD, CYP, ACTH, GST)

The following biochemical parameters were measured in the framework of a routine biological effects monitoring conducted by the German Federal Fisheries Research Center on dab from this sampling area: 7-ethoxyresorufin-O-deethylase (EROD) activity, total protein and levels of cytochrome P450 1A (CYP1A) protein were measured in dab liver (for further analytical details see Kammann *et al.* 2001). From muscle tissue cholinesterase activity (ACHE) was measured colorimetrically according to Ellmann *et al.* (1961). Glutathion-4-S-transferase (GST) activity in liver was determined with the method described by Bressler *et al.* (1999). The total protein content of liver samples was measured according to Bradford *et al.* (1976).

Leukocyte isolation

Media and cells were kept on ice and washing procedures were performed at 4°C. Cell suspensions of head kidney leukocytes (HKL) were prepared by forcing the tissues through a 100 µm nylon screen (Swiss Silk Bolting Cloth Mfg, Zurich, Switzerland). Isolated HKL were washed 3 times with wash medium (10 min, 550 x g) and resuspended in cell culture medium (RPMI-1640 supplemented with 100 000 IU l^{-1}

penicillin, 100 mg l^{-1} streptomycin, 4 mM L-glutamine and 1% [v/v] carp serum (chemicals: Biochrom, Berlin, Germany; carp serum: serum from 15 individual *Cyprinus carpio* L. was pooled, heat inactivated for 30 min at 56 °C, 0.2 µm filtered and stored at -20 °C until use). Numbers of viable cells were determined by trypan blue exclusion in a Neubauer haemocytometer.

Production of reactive oxygen species by head kidney leukocytes

Generation of reactive oxygen species (ROS) by head kidney leukocytes was measured by means of the nitro blue tetrazolium salt (NBT) reduction assay. Cell suspensions were incubated in 96-well flat-bottom microtiter plates (10^6 cells in a final volume of 175 µl of cell culture medium) in triplicate and their ROS production was induced by adding 0.15 mg l^{-1} phorbol myristate acetate (PMA). The indicator NBT was added at 1 g l^{-1}. Wells without PMA served to determine the basal ROS generation of the cells. After incubation for 2 h at 18 °C, the supernatants were removed and the cells were fixed by adding 125 µl of 100 % methanol. Each well was washed two times with 125 µl of 70 % [v/v] methanol. Methanol was removed and the fixed cells were air dried over night and stored in the dark for up to two weeks. The reduced NBT (formazan) was dissolved in 125 µl 2 M KOH and 150 µl DMSO per well (all chemicals: Sigma-Aldrich, Germany). The optical densities were recorded with a spectrophotometer at 650 nm.

Endocytosis activity of head kidney phagocytes

Endocytosis activity of HKL was measured by means of neutral red retention as described by (Mathews *et al.* 1990). This assay was adapted to microtiter plates. Briefly, 10^6 cells were incubated in a final volume of 175 µl culture medium for 2.5 h at 18 °C with 10 mg l^{-1} neutral red (NR, Sigma-Aldrich, Germany). All set-ups were made at least in triplicate. After incubation each well was washed two times with 125 µl of phosphate buffered saline (PBS). After removing the PBS, the cells were air dried over night and frozen at -20 °C for up to two weeks. For spectrophotometric readings the cells were lysed with 100 µl acid ethanol (3 % HCl in 95 % Ethanol) and mixed with 100 µl PBS. The optical densities were recorded at 492 nm.

Lysozyme assay

Lysozyme activity of dab plasma was determined by means of a turbidimetric assay according to Parry *et al.* (1965). A suspension of 0.2 g l^{-1} *Micrococcus lysodeikticus* (Sigma-Aldrich, Germany) in 0.05 M sodium phosphate buffer (pH 6.2) was mixed with 25 µl of dab plasma to give a final volume of 200 µl per well. The optical density was read in a spectrophotometer at 530 nm immediately after mixing, after 0.5 min, and after 4.5 min at a temperature of 20±2 °C. The decrease in absorbance was used to calculate lysozyme activity. One unit of lysozyme activity is defined as the amount of sample causing a decrease in absorbance of 0.001 OD min^{-1}. Hen white egg lysozyme (Sigma-Aldrich, Germany) was used as external standard as described by Hutchinson and Manning (1996).

Statistics

Normality of the data was tested with the Kolmogorov-Smirnow test. To determine the significance of differences between groups, data were compared by Student's t test, Mann-Whitney's rank sum test, or by Kruskal-Wallis ANOVA and subsequent multiple comparison of means using the Student-Newman-Keuls method at a probability of error $p<0.05$. Correlations between data sets were tested with Pearson's Product Moment Correlation test or with the Spearman Rank Correlation test. Correlations were considered as significant at a probability of error $p<0.05$. All calculations were done using the computer programme Sigma Stat (SPSS Science Inc.).

Results

Haematocrit

The haematocrit of fish blood is considered to represent a simple, non specific indicator of overall health (Blaxhall 1972, Anderson 1990). Decreased haematocrits were found in fish with nutritional deficiencies, infections with micro organisms or other health aberrations (Blaxhall 1972). In the dab examined here, regional differences ($p<0.05$) were found between individuals from the North Sea with a haematocrit of 22.5 (18-28) % and individuals from the Baltic with 39 (31-43) % (table 1).

Table 1: Summary of immune parameter of dab (*Limanda limanda*) collected from 6 different sampling sites in the North Sea and the Baltic. ROS: production of reactive oxygen species by head kidney leukocytes with and without (basal) stimulation by the phorbol ester phorbol myristate acetate (PMA). Given are mean and range of the measurements.

Parameter	North Sea (n=289-300)	Baltic Sea (n=32)
Haematocrit (Vol %)	22.5 (18-28)	39 (31-43)
Plasma lysozyme Units ml^{-1}	974 (562-1635)	1254 (925-1899)
Endocytosis OD	0.025 (0.008-0.069)	0.024 (0.007-0.049)
Basal ROS OD	0.119 (0.041-0.556)	0.100 (0.031-0.412)
PMA activated ROS OD	0.721 (0.278-2.207)	0.509 (0.119-1.186)

In both populations, length and age of fish did not affect the haematocrit, while a low, but significant ($R= 0.29$; $p<0.05$) influence of weight was noted. In the North Sea, individuals from JMP had higher haematocrits compared to dab from P02, N04 and N06 sample sites ($p<0.05$) and individuals from P01 had higher haematocrits compared to N04 and N06 dab ($p<0.05$, Fig. 2).

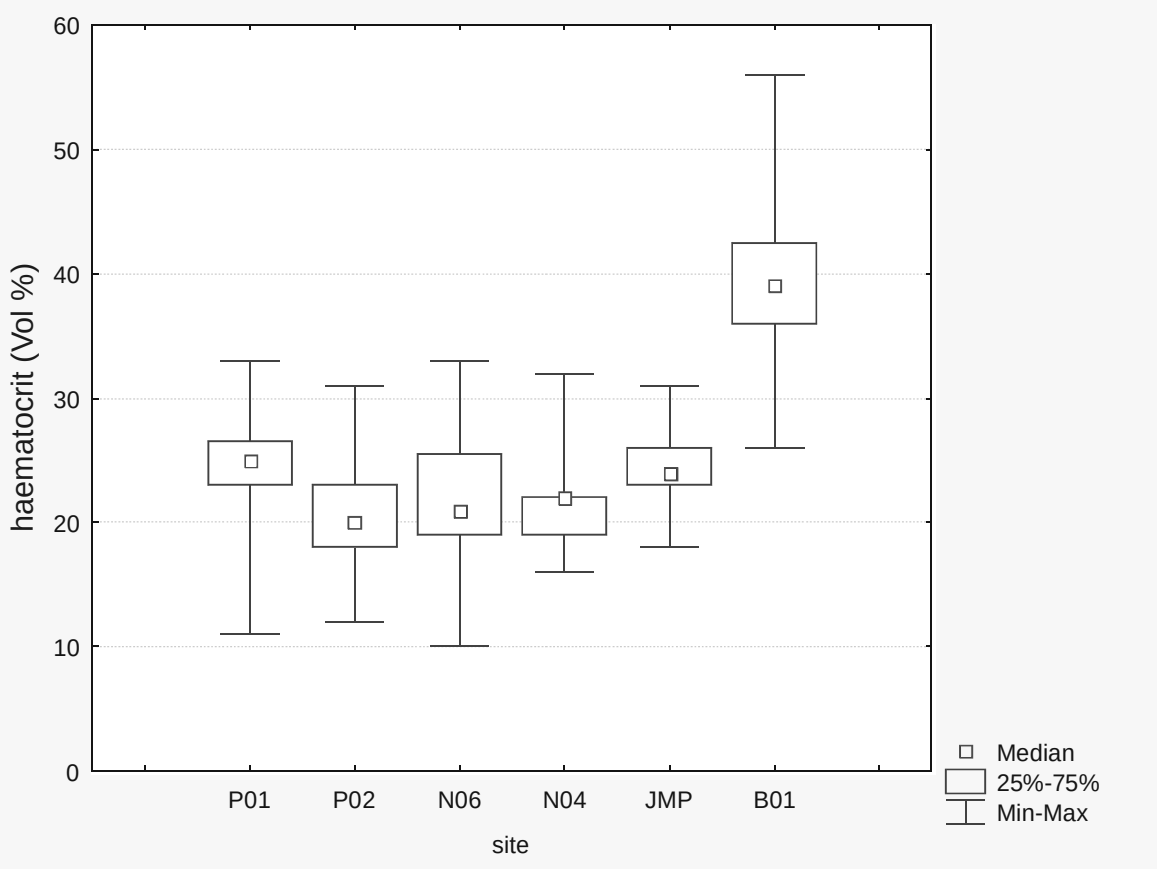


Figure 2: Haematocrit in dab *Limanda limanda* collected at 6 different regions in North and Baltic Sea during 3 consecutive sampling campaigns in 1999 - 2001. For sampling locations see Fig. 1. Dab from the Baltic Sea (B01) had significant higher haematocrits than North Sea dab ($p<0.05$). In the North Sea, haematocrit was significantly higher in dab at P01 compared to individuals at P02, N06 and N04 ($p<0.05$). Dab JMP had also significantly higher haematocrit values than fish from P02, N06 and N04 ($p<0.05$).

Lysozyme

The lysozyme activity in the plasma of North Sea dab was with 974 (562-1635) IU ml^{-1} significantly lower ($p<0.05$) than the plasma of dab caught in the Baltic Sea with an activity of 1254 (925-1899) IU ml^{-1} (table 1). In both populations, plasma lysozyme activity was not affected by weight, length or age of the individuals. Significant differences ($p<0.05$) in activity, however, were found between dab from oil- and gas platforms (P01 and P02) and dab collected from the German Bight (JMP, Fig. 3). Dab sampled at the Dogger Bank (N04) had significantly ($p<0.05$) higher plasma lysozyme levels than dab from P01 and N06 (Firth of Forth) locations (Fig. 3).

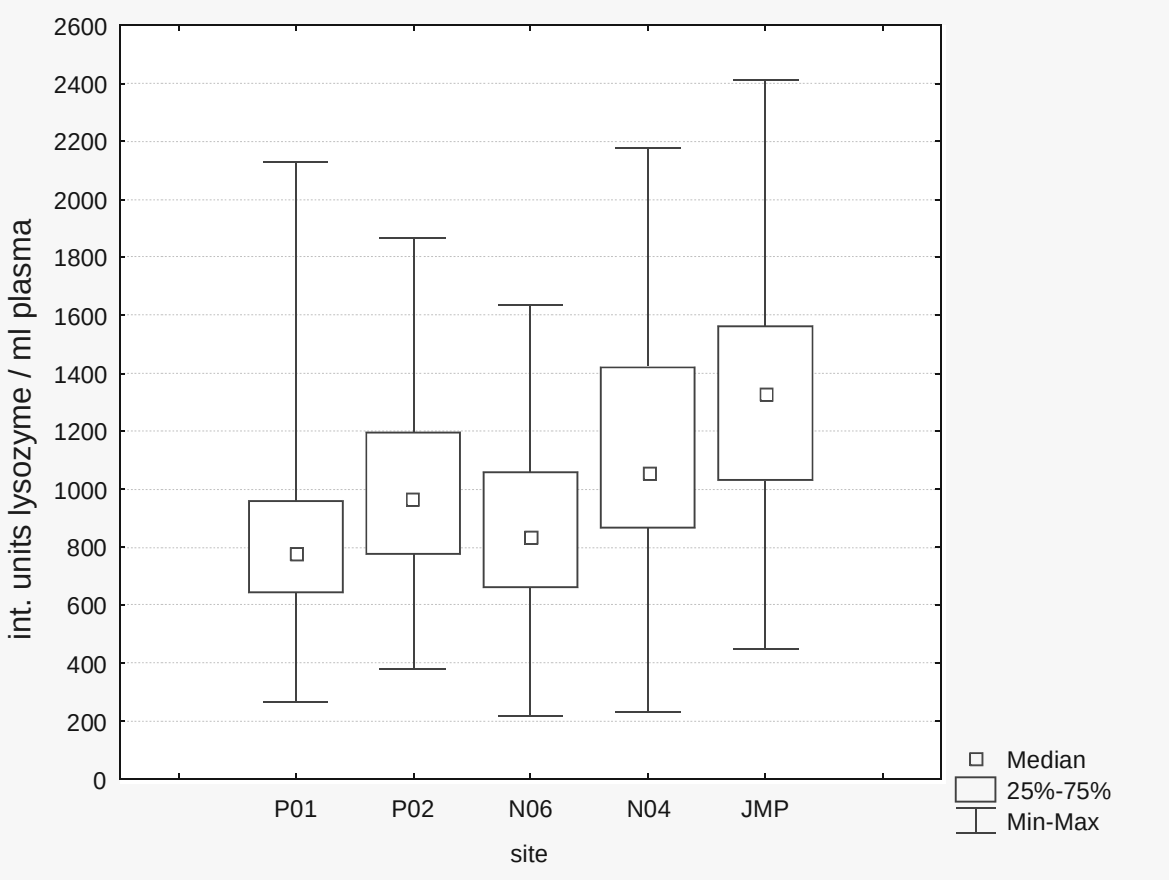


Figure 3: Plasma lysozyme activity in dab *Limanda limanda* collected at 6 different sites in North and Baltic Sea during 3 consecutive sampling campaigns in 1999 –2001. For sampling locations see Fig. 1. Individuals at JMP had significantly higher lysozyme levels compared to dab at P01, N06 and P02 ($p<0.05$) and at N04 individuals had significant higher lysozyme values than dab collected from P01 and N06 ($p<0.05$).

Endocytosis by head kidney phagocytes

The endocytosis activity of head kidney phagocytes was not different in dab from North Sea and Baltic Sea locations (table 1). It was also not affected by age or length of fish, but with increasing weight of the individuals, the endocytosis activity of their head kidney leukocytes increased ($p<0.05$). In the North Sea, head kidney derived phagocytes from dab collected at the JMP station showed a significant higher endocytosis activity when compared to individuals from P02 and N06 ($p<0.05$). In addition, cells from dab collected at N06 had significant lower endocytosis activity than individuals from N04 and P01 ($p<0.05$, data not shown).

Production of reactive oxygen species

The basal ROS production of head kidney derived leukocytes from North Sea dab was not different from measurements obtained from individuals collected at the sampling site in the Baltic Sea. Upon PMA stimulation, cells from individuals collected in the

North Sea responded with higher ROS production ($p<0.05$) than fish from Baltic (table 1). Both basal and stimulated ROS production were significantly influenced by the age of fish (basal ROS production: $R=0.19$, $p<0.05$; PMA stimulated ROS production: $R=0.40$, $p<0.01$). Weight or length had no effect on basal ROS production of HKL, while the PMA stimulated ROS production of HKL was significantly ($R=-0.20$, $p<0.01$) influenced by fish weight.

When dab from different areas in the North Sea were considered, clear differences in ROS production could be observed. Head kidney leukocytes from dab at the Dogger Bank (N04) had significantly higher ($p<0.05$) basal ROS production compared to individuals from the sites near gas or oil platforms (P01, P02) and at the Firth of Forth (N06). Cells obtained from dab collected near oil platforms at P01 also had significantly ($p<0.05$) lower basal levels of ROS production compared to dab from the German Bight at the location JMP (Fig. 4).

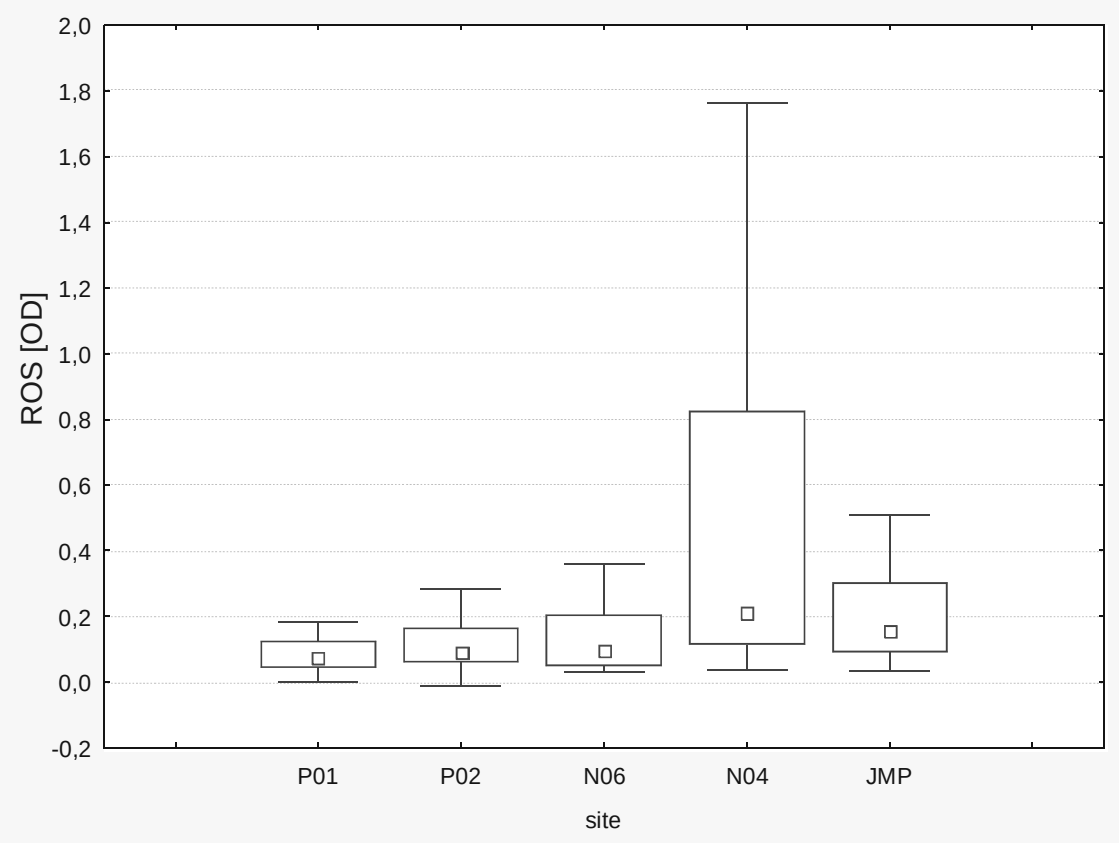


Figure 4: Basal production of reactive oxygen species (ROS) by head kidney cells derived from dab (*Limanda limanda*) at 5 different locations in the North Sea. For sampling locations see Fig 1. Dab from N04 had a significantly higher ROS production compared to individuals from P01, P02 and N06 ($p<0.05$). Head kidney leukocytes from JMP dab also showed a significantly higher ROS production than cells from individuals at P01 ($p<0.05$).

When HKL were stimulated with PMA, the cells responded with increased ROS production and showed slightly higher measurements in dab collected at the stations JMP and N04 compared to fish from the oil and gas platforms. These differences, however, could not be confirmed as statistically significant (Fig. 5).

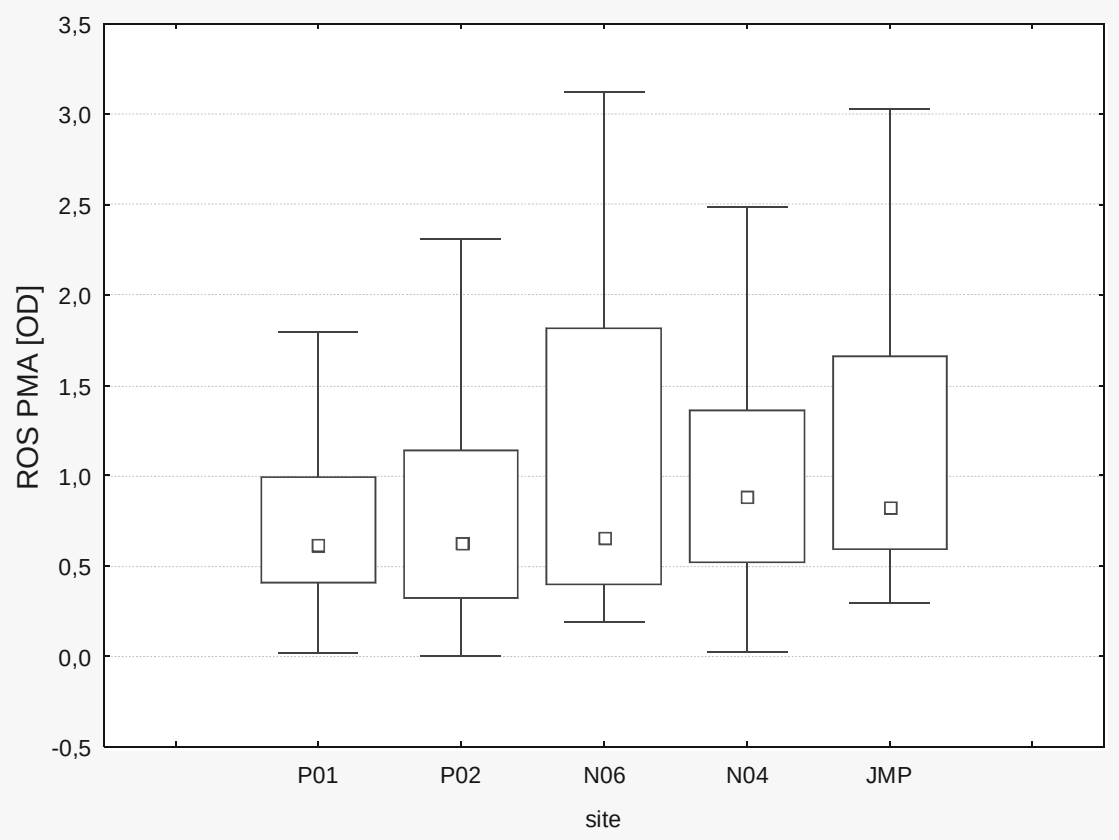


Figure 5: Production of reactive oxygen species by head kidney derived dab (*Limanda limanda*) leukocytes upon stimulation with the phorbol ester PMA. The dab were collected at 5 different sampling sites in the North Sea during 3 sampling campaigns in 1999 - 2001. Statistically significant differences were not found between the sampling locations.

Cross correlation of innate immune responses with physiological biomarkers and grossly visible diseases

Measurements of innate immune responses and the physiological biomarkers applied here were collected from the same individuals. When in a pooled data set plasma lysozyme levels of individual North Sea dab were compared to endocytosis and ROS production of HKL from the same individual, a positive correlation was found for pinocytosis and a negative correlation was seen for PMA stimulated ROS production (see table 2). Dab with decreased haematocrits also had lower plasma lysozyme levels (table 2).

When measurements of the physiological biomarkers applied were compared to plasma lysozyme levels, pinocytosis activity and ROS production of HKL of the same individual, the following correlations were found: dab with induced EROD activity had decreased plasma lysozyme levels and decreased ROS production ($p<0.01$, table 3) and individuals with impaired ROS production of HKL also had lower ACHE activity ($p<0.01$, table 3). Correlations between the haematocrits of individual dab and responses of physiological biomarkers were not found.

The presence or absence of grossly visible diseases or parasites had a marked impact on several of the innate immune parameter measured, but the pattern varied with the disease or infection (see table 4). Individuals with a lymphocystis infection had reduced plasma lysozyme levels with 892 (628-1123) IU ml^{-1} compared to 1007 (797-1337) IU ml^{-1} in non infected fish. In dab with anomalies in pigmentation, haematocrits were decreased with 22 (19-24) % compared to 23 (20-27) % in not affected individuals and basal as well as PMA triggered ROS production of HKL from affected dab was increased with 0.156 (0.0985- 0.387) OD values basal and 1.085 (0.546-1.772) OD values PMA stimulated ROS versus 0.105 (0.062- 0.208) and 0.639 (0.375- 1.169), respectively in not affected individuals. In individuals with skin ulcers or epithelial papillomas, measurements of the innate immune responses monitored here were not different from not affected dab.

Infections with nematodes were accompanied with significantly reduced haematocrits (mean 22, range 19-24 % in infected and 23, range 21-27 % in not infected dab), decreased plasma lysozyme levels (mean: 966 range: 694- 1189 IU ml^{-1} versus 991 (797- 1369) IU ml^{-1}), reduced basal ROS production but increased endocytosis activity of HKL. Infections of dab with gut dwelling acanthocephala were accompanied with reduced endocytosis activity of HKL. Multiple regression analysis indicated that from the immune parameters considered here, haematocrits and pinocytosis activity of HKL mainly were affected by nematode infections, plasma lysozyme levels by lymphocystis infection and ROS production of HKL by anomalies in pigmentation (table 5).

Table 2: Cross-correlation between immune parameters measured in dab (*Limanda limanda*) collected in the North Sea from 4 different locations (see Fig. 1). Spearman's Correlation on Ranks at p: <0.05:*, p<0.01: **: N= 286-300

	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Haematocrit	1	0.18**	0.12*	-0.06	-0.08
Lysozyme	0.18**	1	0.24**	0.06	-0.13*
Pinocytosis	0.12*	0.24**	1	-0.06	-0.10
Basal ROS	-0.06	0.06	-0.06	1	0.50**
PMA activated ROS	-0.08	-0.13*	-0.10	0.50**	1

Table 3: Correlations between biochemical parameters of dab *Limanda limanda* and the immunological parameters applied here. The measurements were taken from the same individuals. The dab were collected in 1999-2001 at 5 different locations in the North Sea (locations see Fig. 1, *N*=84-200). Spearman's Correlation on Ranks, marked are correlations at $p<0.05$: *, $p<0.01$: **; Abbreviations: *EROD*: 7-ethoxyresorufin-O-deethylase assay; *CYP:* cytochrome P450 1A protein concentration; *ACHE*: cholinesterase activity; *GST*: glutathion-4-S-transferase activity. EROD, CYP, ACHE and GST data from T. Lang (German Federal Research Centre for Fisheries, Cuxhaven).

Biomarker	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
EROD	0.00	-0.26**	-0.05	-0.44**	-0.29**
CYP	0.00	0.12	0.08	0.09	0.00
ACHE	-0.10	0.03	-0.06	0.35**	0.39**
GST	0.01	-0.37**	0.16	-0.23*	0.01

Table 4: Comparison of the presence of grossly visible diseases and parasites in dab *Limanda limanda* and immunological parameters measured from the same individual. The dab were collected in 1999 – 2001 at 5 different locations in the North Sea (see Fig. 1). Compared were immune parameters of affected dab to measurements obtained from not infected individuals by means of the Mann-Whitney rank sum test. Listed are p values obtained from the test. Statistically significant differences in the immune response between the groups at $p<0.05$ are marked in **bold**. (+): increased immune parameter in infected dab; (-): depressed immune parameter in infected individuals. n=286-300. *Lymp*: lymphocystis; *Eppap*: epidermal hyperplasia/papilloma; *Ulc*: acute/healing skin ulceration; *Pigmel*: pigmentary abnormality; *Nemato*: nematodes (liver); *Acanth*: intestinal acanthocephalan. Disease and parasite data from T. Lang (German Federal Research Centre for Fisheries, Cuxhaven)

Disease	Haematocrit	Lysozyme	Pinocytosis	Basal ROS	PMA activated ROS
Lymph	0.17	**0.01(-)**	0.18	0.47	0.32
Eppap	0.16	0.61	0.95	0.71	0.10
Ulc	0.17	0.73	0.47	0.19	0.36
Pigmel	**0.01(-)**	0.45	0.58	**0.01(+)**	**0.01(+)**
Nemato	**0.01(-)**	**0.04(-)**	**0.01(+)**	**0.02(-)**	0.69
Acanth	0.12	0.35	**0.02(-)**	0.17	0.64

Table 5: Multiple linear regression between grossly visible diseases and parasites in dab (*Limanda limanda*) and the immune parameter measured in the same individual. The presence of grossly visible diseases or parasites is tested as explaining variable on the immune responses applied here. Given are the p values obtained from the calculation. An influence was considered to be significant at $p<0.05$*; $p<0.001$**. n=286-300. *Lymp*: lymphocystis; *Eppap*: epidermal hyperplasia/papilloma; *Ulc*: acute/healing skin ulceration; *Pigmel* pigmentary abnormality; *Nemato*: nematodes (liver); *Acanth*: intestinal acanthocephalan. Disease and parasite data from T. Lang (German Federal Research Centre for Fisheries, Cuxhaven)

Disease	Haematocrit	Lysozyme	Pinocytosis	ROS	PMA activated ROS
Lymph	0.42	0.04*	0.40	0.20	0.20
Eppap	0.23	0.57	0.91	0.50	0.50
Ulc	0.14	0.72	0.72	0.74	0.74
Pigmel	0.09	0.55	0.18	<0.01**	<0.01*
Nemato	0.04*	0.20	0.02*	0.14	0.84
Acanth	0.86	0.60	0.12	0.72	0.88

Discussion

Numerous studies showed that water pollutants indeed have an impact on innate as well as on adaptive immune responses (recently reviewed by Zelikoff *et al.* 2000, Bols *et al.* 2001). This became very clear when fish were exposed to various substances such as metals, pesticides or insecticides under laboratory conditions and could be confirmed when feral fish were collected from polluted sites. Thus several authors (Dunier and Siwicki, 1993, Wester *et al.* 1994, Zelikoff *et al.* 2000, Bols *et al.* 2001) recommended fish immune assays as indicators to predict toxicological risk associated with pollution in aquatic environments. Especially innate immune responses, which protect an organism against infections without depending upon prior exposure to any particular pathogens, are discussed as good candidates for biomarker as tools for assessing unfavourable biological effects (Wester *et al.* 1994).

In feral fish, biological parameters such as enzyme activities or immune responses underlie natural fluctuations, and when considering these responses as biomarkers or indicators for environmental degradation, pollution mediated effects have to be distinguished from natural fluctuations. Therefore, a sufficiently large number of individuals of comparable size should be collected, most desirable in a long term study (Anderson 1990). Our findings here underline the significance of this notion: In total 336 individuals were analysed during the 3 sampling campaigns in 3 consecutive years, and some variation of the measurements in all parameters were seen between the campaigns (data not shown). In addition, measurements of haematocrit, pinocytosis, basal and PMA stimulated ROS were significantly influenced by body weight and age of dab, even though the analysis was restricted to a defined size class of 20-24 cm. These findings are consistent with results from a field study on flounder (Chapter 5), Japanese medaka (*Oryzias latipes*, Duffy *et al*. in press) and from mammals where sensitivity to toxic insult decreases with increasing age (Parkinson and Safe 1987). In the present study, only female dab were collected during campaigns in August and September in order to reduce seasonal and sex related variations, which were described in detail by Hutchinson and Manning (1996). In addition, North Sea dab were collected at locations with similar characteristics in respect to salinity, temperature and water depth in order to reduce variations caused by these factors. The significance which genetic and/ or habitat differences might have on fish immune responses underline different haematocrits, plasma lysozyme levels and respiratory burst activity of head

kidney leukocytes of North Sea dab compared with individuals from the Baltic (table 1). Therefore, dab from the Baltic Sea were not considered for further analysis herein.

When considering the North Sea sampling locations of the present study, a pollution gradient was discussed by Kammann *et al.* (2001) on basis of PAH contamination of the sediment. Highest contamination expressed as Σ of 16 PAHs was measured at stations P02 with 35.87 ng g^{-1} and N06 with 27.47 ng g^{-1} dry matter, followed by P01 with 13.78 ng g^{-1}. At the stations JMP (6.02 ng g^{-1}) and N04 (5.79 ng g^{-1}) lower levels of PAHs were analysed. Along with this pollution gradient, the plasma lysozyme level and the respiratory burst activity of head kidney phagocytes was reduced in individuals from higher polluted stations (see Figs. 3-5). In contrast, haematocrits and pinocytosis activity of HKL were not altered in dab from regions with increased PAH contamination. These findings substantiate observations from other studies on dab. In individuals caught after a major oil spill in the North Sea, serum lysozyme levels in dab were negatively correlated with the PAH level in the sediment (Secombes *et al.* 1997). Dab exposed to oil-contaminated sediment or sewage sludge had lower serum lysozyme levels (Tahir *et al*. 1993) and decreased ROS production by head kidney phagocytes relative to control groups (Secombes *et al*. 1991, Tahir *et al*. 1993). In vivo exposure of dab to different concentrations of cadmium also was related to a reduction of the ROS production by head kidney phagocytes when compared to unexposed individuals (Hutchinson and Manning 1996b).

The work reported here was part of an integrated field study, which included a simultaneous assessment of other biomarkers recommended by the ICES (ICES 1996), such as the induction of mono-oxygenase ethoxyresorufin O-deethylase (EROD) or glutathion-4-S-transferase (GST) in liver cells, the inhibition of acetylcholin esterase (ACHE) in muscle, and grossly visible fish diseases and parasite infections. EROD is known as a sensitive indicator of the exposure to lipophilic compounds such as PAHs, dioxins and coplanar PCB congeners (Goksoyr and Förlin 1992, Boer *et al.* 1993, Sleiderink *et al.* 1995). Cholinesterase (ACHE) is widely used to estimate neurotoxic impact of pollutants on the cellular level of marine organisms (Galgani *et al.* 1992, Bressler *et al.* 1999) and the induction of glutathion-4-S-transferase (GST) activity indicates an adaptation of the organism to enhanced pollution stress (Bressler *et al.* 1999). In the present study, responses of the innate immune system and these biomarkers were recorded from the same individual dab, which allowed to compare

responses of the different parameters on basis of individual fish. These comparisons showed that dab with induced EROD or GST activities also had lower lysozyme activity and decreased phagocytes responses, which indicates that in fish under pollution stress, several functional systems were affected. The observations of the present study on dab confirm findings on flounder from German Bight (Chapter 5), where in individuals with decreased integrity of hepatocyte lysosomes, the EROD system also was induced and innate immune responses were impaired. In the present study on dab, correlation coefficients between biomarkers and immune parameter were much higher compared to flounder (Chapter 5), most probably because a more pronounced pollution gradient was found between the sampling locations of the present study compared to the locations of the German Bight, where the xenobiota load decreased during the past decade (for details see De Jong *et al.* 1999) and a weak pollution gradient only was found (Schmolke *et al.* 1999). In addition, the present study concentrated on female dab which were collected during the same month in every year, which most probably reduced natural variation of the measurements.

A link between impaired immune functions of fish and diseases susceptibility was suspected for long and formed the basis of programmes on a systematic recording of occurrence and prevalence of grossly visible diseases in dab in the North Sea, which was launched by ICES member countries since 1980 (Dethlefsen *et al.* 2000). In the present study, occurrence and prevalence of grossly visible diseases were recorded along with an analysis of some innate immune responses. Individuals infected with the lymphocystis virus, with nematodes or acanthocephala displayed altered plasma lysozyme or head kidney phagocyte activities compared to not affected dab. In parasite-infected individuals, lysozyme and respiratory burst activities were decreased, while in dab with lymphocystis infection, non specific cellular responses appeared to be not affected, in contrast to observations on American plaice, *Hippoglossoides platessoides* (Fabricius), where head kidney cells displayed enhanced phagocytosis and respiratory burst activity in association with lymphocystis infection (Marcogliese *et al.* 2001). Other diseases, such as epidermal papilloma or skin ulcers were not observed to be associated with altered lysozyme or head kidney phagocyte activity.

Taken together, the data presented here show that in dab plasma lysozyme and head kidney phagocyte activity display differences along with a pollution gradient. Innate

immune responses applied here were altered along with the physiological biomarkers GST or EROD, and with the occurrence of fish diseases such as lymphocystis. Innate immune parameters applied here can be easily integrated into a biological effects monitoring program and will provide supplementary information about immunomodulatory effects associated with exposure to contaminants. Especially plasma lysozyme, which can be analysed in an easy and inexpensive assay, will be a good parameter in a battery of other bioindicators.

Chapter 7

General Discussion

General discussion

Summary and conclusions

In this thesis, innate immune responses of flatfishes were integrated as biomarkers into biological effect monitoring studies. The chosen assays, which previously were stated as sensitive to xenobiotic exposure in other studies (for review see: Bols *et al.* 2001) were: blood haematocrit, lysozyme activity in plasma as well as endocytosis activity and generation of radical oxygen species from granulocytes and macrophages/monocytes. For an implementation of these tests into monitoring programmes under field situations, leucocytes from different organs were tested for phagocyte responses. Head kidney derived leucocyte suspension contained the highest number of responding cells, while other tissues such as spleen or blood harboured minor percentage of these cells. Corresponding to the number of phagocytes, ROS and phagocytosis readings were low from peripheral blood and spleen leucocytes. Thus, head kidney derived leucocytes were used. In order to reduce sampling effort in field studies, these cell suspensions could be used in respiratory burst assays without further enrichment protocols. In addition, lysozyme activity could be recorded from flounder plasma in a simple turbidometric assay.

These assays were applied in two different field studies, where they proved their applicability in the field. Up to 20 individuals could be processed for innate immune assays within a single day and from these individuals sufficient tissue material could be obtained to conduct the described assays, without interfering with other working groups, which had to collect tissues from the same individual as well.

In addition to the immunological measurements, information about the pollution intensity in the sediment and biota as well as the effects of pollution on biota were derived in both studies by chemical, bioaccumulation and biological effect monitoring with existing biomarkers of chemical exposure (e.g. EROD or ACHE). The applied biomarkers to some extent were recommended for marine monitoring by international agencies, such as ICES (ICES 1996, 1999).

In the study on flounder 5 different locations in the German Bight with different anthropogenic impact were sampled over a period of two years. When considering heavy metal and chlorinated hydrocarbon contaminations in sediment, only slight differences were found. Some xenobiotica were detected in all sites, while some were only found in single location, which has also been reported for this region earlier (Broeg *et al.* 1999, Schmolke *et al.* 1999). While no correlation of plasma lysozyme activity

was observed with the concentration of chlorinated hydrocarbons or heavy metals in the sediment, negative correlation of plasma lysozyme activity was seen with standardized contamination load in *Mytilus edulis* collected from the sediments. The contamination load in mussel exhibited a clear site specific pollution gradient, which might reflect the contamination of the food used by flounder at a particular location. The residues in flounder muscle did not reflect the same pollution burden found in the sediment for the same xenobiotica. The sediment or mussel residues reflect site specific contamination levels, while levels found in flounder reflect the pollutant exposure of the individual.

At some locations, the measurements of the immune parameters applied here varied within wide ranges, which made it difficult to establish spatial differences, some general trends, however, could be drawn: The individual contamination pattern found in flounder had immunmodulatory impact on the parameter assessed here. Plasma lysozyme activity was decreased in flounder contaminated with DDT adducts and some PCBs, while cellular functions such as phagocytosis and respiratory burst were stimulated by some chlorinated hydrocarbons. The induction of ROS production in flounder with high PCB load shows the difficulty, by trying to assess the impact of xenobiotics by immunological measurements. Each congener from a chemical group might interfere with immune reactions in a different way. For mammals it is well known, that non-coplanar PCBs increases PMA stimulated ROS production in granulocytes (Narayanan *et al.* 1998; Fischer *et al.* 1998), while coplanar PCB congeners with high affinity for the aryl hydrocarbon receptor (AhR) do not activate neutrophils (Brown *et al.* 1998). In mammalian granulocytes, PCBs may induce an inhibition of superoxide dismutase (SOD) activity, which may result in oxidative stress and thus leads to an imbalance between production of free radicals and antioxidant defense mechanisms of the cell. This in turn can induce tissue damage and hasten the onset of granulocyte apoptosis (Narayanan *et al.* 1998). In flounder the AhR receptor has also been described (Besselink *et al.*1998) and mechanisms of cell activation might be similar to those reported for mammals, as we also found enhanced ROS production in flounder with high PCB 153 residues. The enhanced production of ROS in flounder from a contaminated region is also reported from Broeg (pers. communication), who found enhanced G6PDH activity in phagocytes. The increased G6PDH activity indicates higher oxidative stress in phagocytes, which reflects the strong influence of xenobiotics on immune competent cells.

A correlation analysis revealed not only interrelations between the parameters applied here and some contaminants but also with biochemical parameters, which are used as biomarkers in pollution monitoring: In flounder with decreased integrity of lysosomal membranes of hepatocytes, immune functions were impaired as well. This might indicate that hepatocytes and head kidney leucocytes exhibit a similar sensitivity to pollutants or pollution mediated oxidative stress in a way as described for mammalian cells (Narayanan *et al.* 1998). In addition, plasma lysozyme as well as phagocytosis activity of head kidney cells were impaired when the activity of EROD was induced, where EROD is a known indicator of exposure to the xenobiotics PAHs and planar PCBs (Broeg *et al.* 1999).

In the flounder field study, a high variability of immunological parameters was found at some locations. This variability was concomitant with reports of others (Dizer *et al.* 2001, Schmolke *et al.* 1999, Broeg *et al.* 1999). This inter-fish variability was often observed as a biological effect in ecotoxicology studies *in vivo* and *in vitro* (Chilmonczyk *et al*. 1997; Chilmonczyk *et al*. 1999; Dizer *et al.* 2001; Hansen *et al.* 1999). The high heterogeneity of the results might reflect a different physiological or pathological status of the tested animals and can be assessed as an indicator for toxic impact (Schmolke *et al.* 1999). As shown for mussels by Dizer *et al.* (2001), some animals show a high pathologic response to a model xenobiotic, while others exposed to the same concentration of the toxicant only show low or moderate responses. Results, derived from targets species with a high inter group variability, are difficult to interpret and may mask modulatory effects of xenobiotics. This especially is true for immune responses.

When considering innate immune responses as biomarkers or indicators for environmental degradation, pollution mediated effects have to be distinguished from natural fluctuations. Seasonal effects such as reproduction or hydrological factors can have a modulatory impact on immune responses in fish. In this thesis, a decrease in ambient salinity from 32 to 16 ppm, however, did not result in a redistribution of leucocyte subsets in the head kidney of the euryhaline flounder. Phagocyte functions of head kidney derived leucocytes, such the respiratory burst and pinocytosis activity as well as plasma lysozyme levels were not altered upon the change in salinity. The findings indicate that these parameters are not sensitive to salinity changes in brackish or estuarine environments. As seen in the field study, the seasonal impact on flounder can exhibit significant temporal differences. The influence of seasonality on lysozyme

content with significant low values, associated with reduced sea temperatures, time of spawning and poor condition factor was also observed for North Sea dab (Hutchinson and Manning 1996) and plaice (Fletcher and White 1976). In consequence, sampling strategies should be adjusted in a way that studies should be conducting in comparable seasons, or/and the a base line knowledge about temperature and reaction patterns of innate immune responses must be followed, to extrapolate the natural influences from the parameters measured in in this biomonitoring programme.

In marine environments, moderate infections with ectoparasitic or gut dwelling organisms are widespread. Infections with these parasites might modulate specific as well as innate immune responses of their hosts (Overstreet 1997). In the present investigation, the infection of flounder with different parasites did not result in alteration of those innate immune response considered here. Due to the high variability in prevalence and abundance of parasite species, and the high variability in immune function no correlations could be observed. Our findings indicate that ectoparasitic crustaceans as the most abundant parasites did not influence the immune responses measured here. For the present study, it could be considered that these parameters were not sensitive to parasite infection.

In a second integrated field study, we focussed on female dab (*Limanda limanda* L). The campaigns were carried out over a period of 3 years in the same month and at marine sites in the North Sea with comparable hydrological characteristics. In addition, a more pronounced pollution gradient was found between the sampling locations when this study on dab was compared to the locations in the study on flounder from the German Bight. In the study area of flounder, the xenobiota load had decreased during the past decade (for details see De Jong *et al.* 1999) and a weak pollution gradient only could be established between the sampling locations (Schmolke *et al.* 1999). By minimizing the natural fluctuations we were able to detect the impact of xenobiota on innate immune responses more precisely. This was most evident in spatial differences of lysozyme activity.

In addition, the correlation coefficients between the response of physiological biomarkers and immune parameter were much higher in the study on dab when compared to flounder. This study, however, revealed a clear impact of infection on immune parameters: in dab infected with the lymphocystis virus or with nematodes, lysozyme levels also were reduced.

In conclusion, the results of this study show that xenobiotics found under natural conditions in fish are immunomodulatory. Some xenobiotica modulate immune reactions in comparable manner as physiological reactions like EROD. The xenobiotics interfere with the cell metabolism, can cause cell impairment, which is seen in decreased lysosomes stability and is concomitant with suppressed immune functions. The innate immune parameters analysed in this study show an influence of the xenobiotica, but these can affect immune functions in different ways and depend much more on the individual contamination load in fish than those found in sediment from the sampling site.

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Zusammenfassung

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Die Verschmutzung der Umwelt, insbesondere in aquatischen Ökosystemen, stellt ein großes Problem des 20. Jahrhunderts dar. Eine große Anzahl unterschiedlichster Xenobiotika gelangt dabei meist über industrielle Abwässer in marine und limnische Habitate, und von dort aus auf unterschiedlichsten Wegen in Organismen, in denen sie mit Stoffwechselwegen interagieren oder direkt toxisch wirken können. Auf diese Weise induzieren diese Substanzen Stress in belasteten Organismen, was sich schließlich als abnehmende Abundanz und/oder reduzierte Reproduktionsfähigkeit der belasteten Biota auf organismischer Ebene äußert, und sich somit auf das gesamte Ökosystem auswirken kann. Ein Ansatz diese durch Umweltverschmutzung hervorgerufenen Veränderungen zu erfassen, stellt die Überwachung mit so genannten Biomarkern oder Bioindikatoren dar: molekulare, zelluläre oder physiologische Parameter eines Organismus, der durch die Belastung mit Xenobiotika moduliert wird. Diese Parameter sollten prinzipiell einfach und kostengünstig zu ermitteln sein, auch gegenüber sublethalen Dosen von Giftstoffen, wie man sie in der Umwelt misst, sensitiv reagieren und so einen Effekt der Kontamination auf den Organismus anzeigen. In der vorliegenden Studie wurden Reaktionen des unspezifischen Immunsystems von Plattfischen in ein Programm zum biologischen Effekte Biomonitoring integriert, um den immunmodulatorischen Einfluss von Xenobiotika abschätzen zu können. Dass einzelne Substanzen oder deren Mischungen immunologische Reaktionen von Fischen beeinflussen können, wurde bereits in Laborstudien von anderen Arbeitsgruppen nachgewiesen.

Wir konnten zeigen, dass aus dem Pronephros der Flunder (*Platichthys flesus* L.) gewonnene Zellen optimal zur Gewinnung und Durchführung funktioneller Tests, wie der Sauerstoffradikalproduktions- oder Endozytosefähigkeit von Granulozyten und Monozyten/Makrophagen waren. Im Vergleich zu Leukozytensuspensionen, die aus Blut oder Milz gewonnen wurden, zeigten unangereicherte Kopfnierenzellsuspensionen die höchste Reaktivität. Daher können diese Zellsuspensionen auch bestens in Feldstudien eingesetzt werden.

Um immunologische Reaktionen als Biomarker oder Bioindikator für Umweltbelastungen einsetzen zu können, muss der Einfluss natürlicher Parameter klar vom Effekt der Kontamination zu trennen sein. Natürliche Einflüsse, wie z.B. hydrografische Faktoren können Immunreaktionen im Fisch beeinflussen. Wie in dieser Studie gezeigt, hat eine Erniedrigung der Salinität von 32 auf 16 ‰ keinen Einfluss auf

die Verteilung von Leukozytensubpopulationen in der Kopfniere der euryhalinen Flunder. Die Funktionen von Phagozyten aus der Kopfniere, die als Pinozytoseaktivität und als Fähigkeit gemessen wurde, Sauerstoffradikale zu produzieren, sowie die Aktivität des im Plasma vorkommenden Lysozyms, wurden durch die Salinitätsänderung nicht beeinflusst. Diese Ergebnisse zeigen, dass die hier gemessen Parameter nicht sensitiv gegenüber Salinitätsänderungen in Brackwasser- oder Estuarbereichen sind.

Eine subklinische Infektion von Flundern mit Parasiten hatte ebenfalls keinen Einfluss auf Faktoren des angeborenen Immunsystems. Unsere Ergebnisse ergaben, dass ektoparasitische Copepoden, die die höchsten Abundanzen der gefundenen Parasiten aufwiesen, keinen großen Einfluss auf Immunreaktionen hatten, und diese somit nicht sensitiv gegenüber subklinischen Parasiteninfektionen waren.

In einer integrierten Biomonitoring Studie in der Deutschen Bucht waren die Lysozymaktivität und Sauerstoffradikalproduktion der Leukozyten von einzelnen Schadstoffen signifikant beeinflusst. Des Weiteren wurden signifikante Korrelationen mit vom ICES empfohlenen Biomarkern, die eine Schadstoffexposition anzeigen, wie EROD oder DNA-Strangbrüche, gefunden. Durch die schwachen regionalen Unterschiede in der Schadstoffbelastung der Deutschen Bucht konnten aber keine regionalen Trends anhand der immunologischen Parameter ermittelt werden.

In einer weiteren integrierten Studie an Klieschen (*Limanda limanda* L.) in der Nordsee, in der ein klarer Verschmutzungsgradient zwischen den einzelnen Stationen beschrieben wurde, konnte gezeigt werden, dass die Fähigkeit von Granulozyten und Monzoyten/Makrophagen Sauerstoffradikale zu produzieren in den belasteten Gebieten deutlich verringert ist. Die Lysozymaktivität im Blut ist signifikant erniedrigt in Klieschen von hoch belasteten Stationen, genau wie in Individuen, die mit dem Lymphocystis Virus oder mit Nematoden infiziert waren.

Die hier vorliegende Studie unterstreicht, dass die ausgewählten Immuntests im integrierten Biologischem Effekte Monitoring im Feld eingesetzt werden können und dabei in der Lage sind, generell modulatorische Effekte von Xenobiotika auf Funktionen des Immunsystems von Fischen unter natürlichen Bedingungen aufzuzeigen.

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